Mutation of Genes Affecting the RAS Pathway Is Common in Childhood Acute Lymphoblastic Leukemia

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Abstract

Deregulation of the RAS-RAF-mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) kinase (MEK)-ERK signaling cascade is often caused by somatic mutations in genes encoding proteins which influence the activity of this pathway and include NRAS, KRAS2, FLT3, PTPN11, and BRAF. We report the first comprehensive mutational screen of key exons of these genes in a large cohort of unselected acute lymphoblastic leukemia (ALL) cases at diagnosis (n = 86) and in a more selected cohort at disease recurrence (n = 47) using the sensitive method of denaturing high-performance liquid chromatography. We show that somatic mutations that deregulate the pathway constitute one of the most common genetic aberrations in childhood ALL (cALL), being found in 35% of diagnostic and 25% of relapse samples. In matched presentation/relapse pairs, mutations predominating at relapse could be shown to be present at very low levels at diagnosis using allele-specific PCR, thus implicating the mutated clone in disease progression. Importantly, in primary samples, we show that mutations are associated with activated ERK and differential cytotoxicity to MEK-ERK inhibitors was shown for some patients. Inhibitors of the pathway, which are currently undergoing clinical trial, may be a novel therapeutic option for cALL, particularly at relapse. [Cancer Res 2008;68(16):6803–9]

Introduction

The RAF-mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK)-ERK signal transduction cascade is one of several MAPK pathways that mediate mitogenic and antiapoptotic responses by coupling signals from cell surface receptors to transcription factors that regulate gene expression. The cascade consists of a G protein working upstream of three kinases: a MAPK kinase kinase (MAPKKK) that phosphorylates and activates a MAPK kinase (MAPKK), which in turn activates MAPK. In the ERK pathway, RAS acts as the G protein, RAF as MAPKKK, MEK as MAPKK, and ERK as the MAPK (1–3).

Deregulated RAF-MEK-ERK signaling is commonly found in cancer cells and is often caused by mutations in the RAS family of genes, which result in constitutive activation of the G protein. For example, mutations in NRAS and KRAS2 have long been recognized as a recurring molecular event in childhood acute lymphoblastic leukemia (cALL), with a reported incidence of between 15% and 22% (4–6). However, aberrant signaling of the RAF-MEK-ERK pathway can also arise from mutational events in genes encoding additional proteins affecting this pathway, including FLT3, PTPN11, and BRAF, and these genes too have recently been shown to be mutated in cALL (6–9). The significance of this pathway activation is emphasized by the observation that such mutations are invariably found in a mutually exclusive manner.

Their overall incidence in cALL is not known but may be significant, particularly at relapse, and may be exploited therapeutically. We report the first comprehensive mutational screen of key exons of NRAS, KRAS2, PTPN11, FLT3, and BRAF in a large cohort of unselected ALL cases at diagnosis and in a more selected cohort at disease recurrence. We show that somatic mutations that deregulate the RAS-RAF-MEK-ERK pathway constitute one of the most common genetic aberrations in cALL and are implicated in progression to relapse in some patients. Importantly, we provide evidence for the rational use of inhibitors of the pathway as a potential novel targeted therapy for cALL.

Materials and Methods

Patient samples. Between January 1999 and 2004, 93 children (less than 17 y) presented with ALL while resident in the northern region of England and diagnostic bone marrow samples were available for study from 86 of them. Relapse samples (n = 47) were collected over a longer period (1988–2003). All diagnoses, remission, and relapse events were pathologically confirmed. Studies were performed with permission of the local ethics committee.

Samples were enriched for mononuclear cells and the blast percentage was determined by morphologic examination after Wright’s staining. DNA was extracted and spectrophotometrically quantified using standard techniques.

Cytogenetic and fluorescence in situ hybridization analyses. Cytogenetic analysis was carried out on diagnostic bone marrow using standard procedures. Patients were screened by fluorescence in situ hybridization (FISH) for the presence of TEL-AML1, BCR-ABL fusions, and MLL gene rearrangements, as previously described (10). High hyperdiploidy (51–65 chromosomes with the classic chromosomal gains) was defined by G banding or FISH. Patients with failed or normal cytogenetic results were screened for the presence of hidden high hyperdiploidy using selected centromeric probes (10). Several patients were screened for the presence of CDKN2A deletions and translocations involving the E2A gene using FISH. Normal and failed cytogenetic results were classified as other if found to have a rearrangement by FISH, as indicated in Supplementary Table S1.

Mutational screening. Genomic DNA was extracted from mononuclear cell preparations of bone marrow aspirates using Qiagen Mini kit (Qiagen Ltd) amplified by PCR, and the amplicons were subsequently screened for mutations by denaturing high-performance liquid chromatography (DHPLC) using a Transgenomic Wave machine. Primer sequences, DHPLC...
variables, and the cell lines or patient samples with characterized mutations, which served as positive controls, are shown in Supplementary Table S2.

**Sequencing.** For those amplicons with chromatographic profiles that differed from wild-type (WT), direct sequencing was performed by purifying 100 μL of PCR product using a QIAquick PCR Purification kit (QIagen) with a final elution volume of 30 μL and then sequenced using both forward and reverse primers with the ABI Version 3 BigDye Terminator Cycle Sequencing kit and analyzed on an ABI Prism DNA sequencer (Applied Biosystems). To characterize minority PCR species, PCR products were subcloned into the pGEM-T Easy plasmid according to the manufacturer's instructions, transformed into *Escherichia coli* (JM109), and isolated plasmid DNA from positive transformants was then sequenced with M13 forward and reverse primers. Sequence alignments were carried out using Omiga software (Accelrys).

**Allele-specific PCR.** Forward primers were designed to specifically amplify KRAS2 and NRAS mutations and PCR conditions were optimized to ensure that only mutant and not WT alleles were amplified. Cell lines with the relevant mutations served as positive controls. PCR products were analyzed by agarose gel electrophoresis and visualized using ethidium bromide. Details of primer sequences are given in Supplementary Table S3.

**Western blotting.** This was carried out using standard techniques. Briefly, whole-cell lysates were prepared using cell lysis buffer supplied by Cell Signaling Technology (NEB). Protein (20 μg) was fractionated using 4% to 20% SDS-polyacrylamide gels (Lanza) and transferred onto polyvinylidene difluoride membrane (Bio-Rad). Immunoblots were probed for phosphorylated ERK (pERK E-4) and ERK2 (K-23) using antibodies supplied by Santa Cruz Biotechnology (Autogen Bioclear). α-Tubulin (Sigma) was used as a loading control and blots were visualized using horseradish peroxidase-conjugated immunoglobulins (Dako) followed by ECL-Plus detection (Amersham). The resulting blots were exposed to Kodak Medical X-ray film (GRI) and scanned using a Fujifilm LAS-3000 image analysis system. Densitometry was carried out using AIDA image analysis software.

**Cytotoxicity assays.** PreB697 cells, maintained in standard culture conditions, or cryopreserved patient blasts were thawed and assessed for viability using trypan blue dye exclusion. All patient samples showed a viability of >80%. Cells were plated out in duplicate at 2,000 cells per well and treated with 0.5 to 10 μmol/L of U0126 (Promega) or 2 to 20 μmol/L of PD98059 (Merck) plus a control vehicle. Following a 96-h drug exposure, cytotoxicity was assessed using the CellTiter 96 Assay kit (Promega), which assesses the capacity of cells to reduce formazan and thus is a measure of metabolically active cells. The resulting absorbances were averaged and expressed as a percentage of the control vehicle. Survival curves were plotted using GraphPad Prism software.

**Statistical analyses.** Student’s *t* tests and contingency tables were calculated using GraphPad Prism software.

## Results

Mutations of genes affecting the RAS-RAF-MEK-ERK pathway are highly prevalent in cALL both at presentation and at relapse. Mutational screening by DHPLC of key exons of *NRAS*, *KRAS2*, *PTPN11*, *FLT3*, and *BRAF* was performed in the cohort of cALL samples. Cell lines or patient samples with well-characterized mutations were used as positive controls. By spiking mutant cell lines or patient samples with characterized mutations, which served as positive controls, are shown in Supplementary Table S2.

Details of mutations and clinical features are summarized in Table 1. Mutations identified in *NRAS* and *KRAS2* predominantly affected codons 12, 13, and 61, although one patient had a KRAS2 L19F mutation. The three *PTPN11* mutations, two found at presentation and one at relapse, included G60V, N58S, and T507K. The three *FLT3* mutations identified at presentation were all novel insertion deletion mutations affecting a similar region of the juxtamembrane region, two yielding size alterations (Fig. 1D). The mutation found at relapse was the well-characterized D835E mutation, sited in the kinase domain of the protein.

The incidence of combined mutations or of the most prevalent (i.e., *NRAS* or *KRAS2*) was not statistically significant between presentation and relapse cohorts (*P* = 0.27, 0.12, and 0.53, respectively, by χ² test). Whereas at relapse the mutated blasts were

![Figure 1](https://cancerres.aacrjournals.org/article-figures/6804/F1.png)

**Figure 1.** A, normalized DHPLC chromatograms of *NRAS* exon 2 amplicons generated from WT cells added to IM-9 cells bearing the G61X mutation. Bar charts showing the incidence of mutations at diagnosis (B) and at relapse (C). *D*, novel FLT3 insertion/deletion mutations.
always the predominant population, in 7 of the 30 diagnostic cases, direct sequencing of PCR products known to have aberrant chromatographic profiles showed no apparent mutation. However, after cloning of the product, sequence data revealed low-level mutations and, given the high blast count in these samples, suggest that only a minor subpopulation of blasts housed these mutations (Supplementary Table S4). These included NRAS and KRAS2 mutations and all of the FLT3 insertion/deletion mutants.

In 26 mutation-positive patients, bone marrow samples taken during clinical remission served as a source of constitutive DNA, and in 25 of them, DHPLC analyses of the appropriate amplicon confirmed that the mutation was somatic and only present in the leukemic blasts. However, in one patient with a N58S alteration in the PTPN11 gene at diagnosis, this same mutation was also evident in the germline, a finding associated with the autosomal dominant, dysmorphic disorders Noonan and Leopard syndromes (11, 12). Mutations in RAS are associated with high hyperdiploidy and a low white cell count. Correlation with mutations status and cytogenetics subgroups revealed a nonrandom distribution with a predominance of mutations in the high hyperdiploid group, showing an incidence of 58% ($P = 0.01$, Fisher’s exact test; Fig. 2A), and consequently, there was also a significant association

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*Mutations not previously identified in cALL.

Table 1. Clinical features of patients and the mutations identified
for presenting white cell count (WCC), with a mean of 19.03 \times 10^9/L for mutated compared with 50.98 for WT (P = 0.01, Student's t test; Fig. 2B). There was no correlation with the presence of mutations and age at diagnosis (P > 0.9, Student's t test) or gender (P > 0.8, Fisher's exact test) or initial response to therapy as measured by bone marrow day 8 blast count (P > 0.34, Student's t test; Fig. 2C). Within the hyperdiploid group, mutation status had no influence on WCC or day 8 blast count (Fig. 2B and C; P > 0.75 for both).

In the TEL-AML1 subgroup (n = 19), there seemed to be a weak correlation with WT TEL status in that 3 of 7 patients who retained WT TEL showed mutations, contrasting with 0 of 10 cases with WT TEL deletion (P = 0.051, Fisher's exact test). In two cases, WT TEL status was inconclusive and they were thus excluded from the analyses.

**Mutations at relapse can be detected in a minority population at diagnosis.** For five relapse samples that housed mutations, matched presentation samples were available for further study. DHPLC analyses of the appropriate amplicon showed WT chromatograms of the corresponding presentation sample in four of the five cases (e.g., Fig. 3A). In three of the four, the more sensitive technique of allele-specific PCR was successfully established to track low-level mutated species, which may be present below the limits of detection of DHPLC. These included KRAS2 G12D, G13D, and NRAS G12V. Using cell lines or patient blasts bearing these specific mutations spiked into WT cells, the sensitivity of each allele-specific PCR was found to be between 0.1% and 1% (data not shown). In both KRAS2 allele-specific PCR, evidence for low-level mutated cells bearing the specific mutation identified at relapse was clearly seen in the presentation but not remission samples (e.g., Fig. 3B). There was no evidence of NRAS G12V mutated cells in the corresponding presentation sample (a summary of results is shown in Fig. 3C).

![Figure 2](image1.png)  
**Figure 2.** Bar chart showing the number of patients with (light) and without (dark) mutations in the principal cytogenetic subgroups (A) and scatter plots comparing presenting WCC and day 8 bone marrow (BM) blast percentages in mutant and WT of the whole diagnostic cohort (B) and within the high hyperdiploid (HD) group (C). Karyotypes of the 23 patients defined as “other” are given in Supplementary Table S1.

![Figure 3](image2.png)  
**Figure 3.** A, DHPLC chromatograms of KRAS exon 1 in patient IPN L139 at presentation, remission, and relapse. B, agarose gel electrophoresis of PCR products generated with KRAS2 exon 1 or allele-specific KRAS2 G12D primers in the same patient. C, table summarizing DHPLC and allele-specific PCR data in the cohort of patients with mutations at relapse and a matched presentation sample.
Clinical samples with mutations are associated with pERK and are sensitive to MEK/ERK inhibitors. Diagnostic material from five patients with and five without mutations was analyzed for the presence of pERK in relation to ERK2 and also to α-tubulin to control for any possible variation in ERK expression (Fig. 4A and B). All five mutation-positive patients had evidence for activated pERK with significant levels seen for the most commonly found NRAS and KRAS2 mutations and a lower pERK level for the less common L19F KRAS2 mutation. By comparison, only one from five of the WT patients showed pERK \( (P = 0.048, \text{Fishér's exact test}) \). For this patient, activation of the pathway is presumably due to an alternative mechanism other than a gain-of-function mutation in the genes examined.

Cytotoxicity assays performed with the MEK/ERK inhibitors PD98059 and U0126 showed a cytotoxic effect in three from four and two from four RAS mutant, pERK-positive samples, respectively (Fig. 4C). No cytotoxicity was seen with any of the four WT, pERK-negative leukemic samples. The NRAS mutant-positive leukemia cell line PreB697, which bears an NRAS G12D mutation but is WT for other components of the pathway, was similarly treated and examined for pERK inhibition. There was complete inhibition of pERK by 10 \( \mu \text{mol/L} \) U0126 (Fig. 5A) and 20 \( \mu \text{mol/L} \) PD98059 (data not shown) after both 8 and 24 h of incubation and a cytotoxic effect was seen for both compounds (U0126 data are shown in Fig. 5B).

Discussion

Activating mutations of the RAS/RAF/MEK/ERK signaling pathway cause growth factor–independent proliferation of hematopoietic progenitors, and in mouse model systems, both oncogenic KRAS and NRAS can act as initiating oncogenes in the induction of myeloid leukemia (13–15). Using a sensitive mutational screening approach of key genes that activate this signaling cascade, we have
found that 35% of an unselected, representative cohort of patients at diagnosis has mutations in NRAS/KRAS2/PTPN11 or FLT3 and is thus one of the most common groups of genetic aberrations found in cALL. Mutations were invariably mutually exclusive, a fact that emphasizes the importance of activation of this pathway in leukemogenesis. Mutations were found in ~60% of high hyperdiploid cases and support the suggestion that such mutations serve as additional, cooperative genetic events in this subgroup of patients (4). A similar study by Paulsson and colleagues (16), but which focussed exclusively on hyperdiploid ALL, found a considerably lower combined incidence of 33%. This difference was most marked for NRAS and KRAS mutations and, as the authors discuss, may be attributable to the different sensitivities of the mutational screening methods used (i.e., direct sequencing versus DHPLC). The similar incidence and spectrum of mutations at presentation and relapse suggests that activation of the pathway per se is not necessarily associated with a poor prognosis and we did not show an association with mutation status and early treatment response, as gauged by day 8 blast levels. Similarly, the study by Paulsson and colleagues (16) found no correlation between pathway mutations and relapse within their hyperdiploid cohort. However, an important finding from this study is that the degree of pathway activation varies depending on the specific mutation and readout of ERK activity may have more prognostic relevance.

Although mutations at relapse were predominant in the leukemic blasts, several diagnostic samples were found to have NRAS, KRAS2, and FLT3 mutations in minority subpopulations, identified only after cloning. These findings concord with the concept of genetic heterogeneity of leukemic blasts at diagnosis, based on studies that provide evidence for the presence of multiple immunoglobulin rearrangements and of minority populations with low-level p53 mutations (17, 18). These heterogeneous leukemic clones can differ in terms of drug sensitivity and more chemoresistant clones may be selected for during therapy and predominate at relapse. The finding that specific RAS mutations, characteristic of the leukemic clone at relapse, can be found at very low level in the matched diagnostic sample is further evidence for the heterogeneity model and suggests that in some patients RAS-mutated clones may contribute to relapse.

The incidence of NRAS and KRAS mutations identified in this study at diagnosis in consecutive children presenting in our region is 31% and is in the higher range of other studies and is almost certainly due again to the increased sensitivity of the DHPLC methodology used (4–6, 19). There are no previous studies at relapse but our finding of a 21% incidence means that RAS mutations are the most prevalent genetic abnormality seen at this stage of disease. The similar incidence at diagnosis and relapse implies that their presence is not a significant risk factor, and whereas early studies suggested an association with higher risk of relapse, a recent study of children treated with more intensive, contemporary therapy found no association of RAS mutation with an adverse clinical outcome (5). One patient in our study had a KRAS mutation at codon 19, specifically an amino acid substitution of leucine for phenylalanine (L19F). In a Caenorhabditis elegans model, this same mutation in H-RAS was associated with an activated RAS phenotype due in part to its effect on the intrinsic rate of GTP hydrolysis (20); however, the level of activated ERK in leukemic blasts bearing this mutation was considerably less than that of the more classic NRAS and KRAS mutations examined. Interestingly, in the Catalogue of Somatic Mutations in Cancer (21), of 8,411 KRAS2 mutations identified from a total of 31,933 tumor samples screened, only 3 possessed the L19F mutation. The tumor type in all three cases was childhood B lineage ALL (6). The explanation for this apparent ALL-specific mutation is not clear at present.

PTPN11 encodes SHP2, a protein tyrosine phosphatase that positively controls RAS function (22). Our study found three PTPN11 mutations, G60V, N58S, and T507K, which have been documented in other tumor types but only G60V has been previously identified in cALL (6, 23). The incidence at presentation compares well with two other previous studies (6, 24), and we show a similar incidence at relapse. Four FLT3 mutations were identified in total, with an incidence of 10% at diagnosis and 2% at relapse, which is in keeping with other studies (7, 8, 25–27). One patient had the characteristic D835E mutation, whereas three others had insertion/deletion mutations of the juxtamembrane region. Small deletions of a 10–amino acid region of FLT3, involving amino acids Tyr299 to Tyr309, have been shown to cause constitutive activation of the protein and it is this region which is affected in all three patient samples (28). Interestingly, one of these involved a deletion/insertion event, which resulted in replacement of 16 nucleotides, thus without yielding a size alteration and consequently was only detectable under DHPLC conditions. A recent report suggests that ALL blasts bearing FLT3 mutations are particularly sensitive to CEP-701, a small-molecule FLT3 inhibitor and thus may be a novel targeted agent for this subgroup of patients (29).

We found no mutations in BRAF, which is in contrast to a recent study by Gustafsson and colleagues (9), in which 6 from 29 selected diagnostic cALL possessed mutations in the kinase domain of BRAF. Some of those identified are known to have an elevated kinase activity and directly signal to ERK by phosphorylating MEK.
and are transforming in NIH3T3 cells (30, 31). The discrepancy with our data set may be due to bias within the patient groups, differences in ethnicity, or possibly a failed detection by DHPLC. The fact that DHPLC routinely detects the V600E mutation that is in close proximity to the other mutations and this DNA region is predicted to be partially denatured by WaveMaker software at the running temperature used (data not shown) makes the latter possibility unlikely.

The clinical challenges remaining in cALL are to further improve cure rates and to achieve cure with minimal toxicity. For those children who relapse, therapeutic options are limited and there is a need for the introduction of more novel drugs. Molecular characterization of primary leukemic blasts has revealed a significant prevalence of mutations, which activate the RAS/RAF/MEK/ERK pathway both at diagnosis and at relapse. A similar mutational screening study may also be warranted in adult ALL because 34.5% of newly diagnosed adult ALL samples have been shown to have constitutive ERK1/2 activation (32).

We also show that primary blasts with classic RAS mutations have activated pERK and those with the highest levels were differentially sensitive to MEK-ERK inhibitors. These data suggest that novel drugs, which target MEK and potentially even RAS, and which are now entering clinical trial for several malignancies, may prove extremely useful agents in cALL, particularly at disease relapse (33–35).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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