Mutations of the ras Proto-Oncogenes in Childhood Monosomy 7

By Andreas Neubauer, Kevin Shannon, and Edison Liu

ras gene mutations are the most frequent molecular changes found in the preleukemic syndromes of adults and may play a role in initiating these diseases and in their progression to acute leukemia. However, little is known about the incidence or importance of these genetic mutations in childhood myeloproliferative states (MPS). The bone marrow (BM) monosomy 7 syndrome accounts for a large percentage of childhood MPS. Although the duration of the MPS is quite variable, children with monosomy 7 eventually develop acute myeloid leukemia (AML). We investigated 20 children (13 with MPS, 7 with AML) with BM monosomy 7 or 7q− for the presence of ras gene mutations using the polymerase chain reaction and hybridization with mutation-specific oligonucleotides. Mutations of N-ras and K-ras were detected in three children. Two patients carrying a ras mutation were in the myeloproliferative phase, and one had acute leukemia. All three patients with ras mutations either died of their disease or relapsed after BM transplantation as compared with 8 of 17 without ras mutations. However, this difference is not statistically significant (P = .14, not significant). We conclude that ras mutations are observed in childhood monosomy 7, though less frequently than in adult MDS, and may play a limited role in the progression of this disease to acute leukemia. More patients are needed to address the prognostic role of ras mutations in this rare disease.

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MUTATIONS IN ONE of the three ras proto-oncogenes Ha-, K-, and N-ras have been observed in a variety of human malignancies.1 In adult hematopoietic malignancies, mutations of the N-ras proto-oncogene predominate2-5 with the frequencies of these mutations ranging from 0% in the chronic phase of chronic myelogenous leukemia (CML)5,6 to 70% in 60% in certain subtypes of myelodysplastic syndromes (MDS) such as chronic myelomonocytic leukemia (CMML).4,7 When all subtypes of MDS are taken together, mutations in the ras proto-oncogenes are the most frequent molecular abnormality in MDS and are seen in approximately 30% of cases. When identified in adults with MDS, ras mutations appear to be predictive of early progression to acute myeloid leukemia (AML).8

A number of recurring, nonrandom cytogenetic abnormalities are seen in both myeloproliferative states (MPS) and AML. Deletions of all or part of chromosomes 5 and 7 are especially common (5q−, 7q−, monosomy 5, or monosomy 7). In adults, these chromosomal abnormalities are frequently associated with prior exposure to alkylating agents and portend a poor prognosis. There is some evidence that the loss of one chromosome 5 is an early molecular event in this disorder and that lesions in ras appear to participate in the progression to acute leukemia.9 The childhood monosomy 7 syndrome is a rare disease that originates in a pluripotent stem cell,10 is clinically characterized by a hypercellular bone marrow (BM) (myeloproliferation), an increased risk for bacterial infections,1 and eventual progression to acute leukemia.11,12 Unlike other childhood myeloproliferative syndromes, some cases of childhood monosomy 7 syndrome are familial.13-15 There are no data on the occurrence or the clinical importance of ras mutations in childhood monosomy 7. To determine possible interactions between deletions in chromosome 7 and mutations in ras, we used the polymerase chain reaction (PCR) to amplify ras sequences from 20 BM samples obtained from children with monosomy 7. PCR amplification products were analyzed for nucleotide substitutions with mutation-specific oligonucleotides and by direct sequence analysis. Our results suggest that lesions in the ras genes are relatively uncommon in childhood monosomy 7 and are not necessary for progression into AML.

MATERIALS AND METHODS

Patients. The patients involved in this study were 20 unselected, consecutive children seen in several referral hospitals across the country. Eighteen patients had monosomy 7, and two patients the 7q− syndrome (nos. 2 and 9). Because the region of overlap for the deletion is on the long arm of chromosome 7,16 the two patients were included in our further analysis. Diagnosis was made using standard hematologic procedures such as examination of the BM and peripheral blood, cytogenetic analysis, and clinical observation. Clinical data were provided by the referring physicians. Several of the patients in this study were siblings similarly affected (case nos. 1A and 1B, and 7A and 7B, respectively) and were the subjects of separate reports.17,19

PCR procedure. DNA from BM cells was extracted using standard procedures.17 The PCR was performed as described elsewhere with some modifications. Briefly, 200 to 500 ng of high molecular weight DNA was used to amplify the areas surrounding codons 12/13, and 61 of N-ras, K-ras, and H-ras, respectively. The primers for the PCR reaction (final concentration 0.5 μmol/L) were from Clontech (Palo Alto, CA). The PCR was performed in 100 mL (10 mmol/L Tris-Cl, pH 8.3, 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.001% [wt/vol] gelatin [Sigma, St Louis, MO], 200 μmol/L of each dNTP [Boehringer Mannheim, Indianapolis, IN; pH 7.0]). 2 U AmpliTaq polymerase [Cetus]) for 35 cycles in an automatic DNA sequencer.

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thermocycler (Perkin-Elmer). In the first cycle denaturing was performed at 94°C for 5 minutes, annealing at 55°C for 1 minute, extension at 72°C for 1 minute. In all other cycles, denaturing time was 1 minute, but with similar cycle times and temperatures as in the first cycle. After PCR was completed, 5% to 10% of the reaction was electrophoresed on a 3% NuSieve (FMC, Rockland, ME)/1% agarose gel.

Oligonucleotide hybridization. The remaining 90 μL of the PCR reaction was denatured with 150 μL 0.4 N NaOH, heated to 95°C for 2 minutes, renatured with 200 μL 2 mol/L Tris pH 7.5, and slotted on nylon filter (Hybond N) paper according to the manufacturer (Amersham, Arlington Heights, IL). DNA was crosslinked using UV light. The oligonucleotide probes covering all possible mutations and the wildtype sequence were from Clontech (Palo Alto, CA). For initial screening, a mixture of three to six specific oligonucleotides (each probe, 7 pmol) was end-labeled with T4 polynucleotide kinase and γ-adenosine triphosphate (ATP) (NEN).

The filters were then hybridized overnight in 5X SSPE (NaCl, NaH2PO4, EDTA), 5X Denhardt's, 100 mmol/L sodium pyrophosphate, 0.5% sodium dodecyl sulfate (SDS), and washed as previously described. The filters were exposed for one to several hours to a XOMAT film (Kodak, Rochester, NY) at −70°C using intensifying screens. To confirm the specific mutation, ras-positive cases were reamplified and either analyzed by oligonucleotide hybridization, or by direct sequencing as described previously. For the Southern transfer, 7 μL of the PCR reaction was electrophoresed using a minigel with two combs set up in parallel. Transfer was performed in a vacuumblotter (Hoefer, San Francisco, CA) onto Hybond-N membranes. After the transfer was completed, DNA was crosslinked using UV light. The filter was cut into two strips and probed. For direct PCR sequencing the PCR products were eluted from a low melting 4% NuSieve gel, extracted with warm phenol, chloroform, and precipitated with ethanol. The water. Seven microliters of this mixture was denatured in the presence of 1 pmol of labeled sequencing primer (an "internally nested" primer) at 95°C for 2 minutes, renatured with 1 μL 0.1 mol/L dithiothreitol (DTT), 2 μL water, and 2 μL 1.8 diluted Sequenase 2.0 (USB, Cleveland, OH) were added. Added into 2.5 μL of the four termination mixtures (dNTP 80 μmol/L, ddNTP 16 μmol/L) was 3.5 μL of this reaction and then incubated for 8 minutes at 37°C. After addition of 4 μL stop solution, 2.8 μL were electrophoresed on an 8% polyacrylamide 50% urea gel run at 80 W. The gel was then exposed to a XOMAT film with an intensifying screen for 1 to 2 days.

The sensitivity of our detection methods is 10% at the cell level using oligonucleotide hybridization, and 25% using direct sequencing of PCR products.14,15

RESULTS

The clinical data and molecular findings of the 20 children with monosomy 7 and 7q−syndrome are shown in Table 1. We demonstrated ras mutations in three cases. These cases included one mutation in codon N-ras 12 (case no. 3), one in codon K-ras 12 (case no. 4), and one in codon K-ras 13 (case no. 5) (Figs 1 and 2). The specimen from patient no. 5 was obtained when he was 6 months old. This mutation could not be found in the DNA extracted from phytohemagglutinin (PHA)-stimulated lymphocytes from the patient (Fig 3, lane 2), nor in DNA from peripheral mononuclear cells from the mother (Fig 3, lane 3), or the father (Fig 3, lane 4). This finding confirms that the ras mutation occurred as a somatic event. Mutations in case nos. 4 and 5 were corroborated by reamplifying the sample and direct sequencing of the PCR products (data not shown). Case no. 3 was confirmed by reamplifying the sample and hybridizing with individual probes. Clinical follow-up data were available in all 20 patients. Eight children received BM transplants (BMT). Because BMT alters the natural history of the disorder,16-18 it confounds efforts to assess the prognostic value of a ras mutation in childhood monosomy 7. Eight of 17 patients without ras mutations, and three of three with such a mutation died of their disease or relapsed after BMT (P = .14, not significant, Fisher’s exact test). The average time of observation after diagnosis was 15.7 months in the three patients with ras mutations versus 34.9 months in those without mutations. In addition, both patients with ras mutations treated with allogeneic BMT relapsed compared with three of seven without ras mutations (Table 1).

DISCUSSION

Preleukemic states are rare in the pediatric age group. This group of disorders includes what has been termed juvenile chronic myelogenous leukemia (JCML), childhood monosomy 7 syndrome, the transient myeloproliferative disorder seen in Down’s syndrome, and a number of nonspecific entities. JCML and monosomy 7 are the most common and best characterized syndromes. There is a considerable clinical overlap between these two disorders, and the outcome is uniformly poor in both. A unique feature of certain pediatric patients with monosomy 7 is its occurrence in siblings.19,21 Molecular investigation of familial monosomy 7 has shown that the genetic predisposition is

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Patients no. 2 and 9 have 7q−, all others monosomy 7. Patients no. 1A and 1B, and 7A and 7B are siblings.

*Patient relapsed after BMT.
not linked to chromosome 7.\textsuperscript{13} Therefore, other genetic aberrations must be involved in the disease. Because of possible interactions between deletions in chromosome 7 and mutations in the \textit{ras} proto-oncogenes documented in adult MDS patients,\textsuperscript{4} we examined the frequency and importance of \textit{ras} lesions in childhood monosomy 7.

We studied an unselected group of 20 consecutive children with MPS or AML and monosomy 7 or 7q\textsuperscript{-}syndrome including four with familial monosomy 7 (Table 1). We demonstrated \textit{ras} mutations in 15\% of patients with childhood monosomy 7 and detected them in both the preleukemic and the leukemic phases of the disease. This finding implies that aberrant \textit{ras} alleles play a limited role in the progression to acute leukemia and are not necessary concomitants to this progression. It is interesting that we found mutations of \textit{K-ras} and \textit{N-ras}, whereas the predominant \textit{ras} gene altered in hematopoietic malignancies is \textit{N-ras}.\textsuperscript{2} However, Liu et al have reported that \textit{K-ras} mutations are common in adult MDS,\textsuperscript{21} suggesting that childhood monosomy 7 shares molecular and clinical features with adult cases of MDS associated with chromosome 7 deletions.

Adult MDS patients with \textit{ras} mutations appear to have a worse prognosis than those without aberrant \textit{ras} alleles.\textsuperscript{6} Assessment of the prognostic importance of \textit{ras} mutations in our patients is confounded by the fact that (1) 45\% of the patients underwent BMT and may have been cured of their hematologic disorder\textsuperscript{19,20}; and (2) the numbers investigated in our analysis are too small to draw a final conclusion. Because the presence of \textit{ras} mutations portends a worse prognosis in adult MDS,\textsuperscript{6} and in childhood ALL,\textsuperscript{15} it is possible that lesions in \textit{ras} may impact in this childhood hematopoietic disorder as well. Clearly, more patients will have to be investigated to properly address this question.

One of our patients, case no. 5, was diagnosed at the age of 6 months with a myeloproliferative syndrome associated with monosomy 7 and a mutation in \textit{K-ras} codon 13. To our knowledge, this is the youngest individual in whom a \textit{ras} mutation has been detected. DNA extracted from the father and mother of this child did not show a mutation in

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**Fig 1.** Detection of \textit{N-ras} mutations in one of three patients with monosomy 7. The PCR products were slotted and probed with: (A) \textit{N-ras} codon 12 probe specific for glycine (wt); (B) a mixture of six \textit{N-ras} codon 12 probes covering all possible mutations; (C) \textit{N-ras} codon 12 probe specific for aspartate. Patient no. 3 shows an ASP mutation in \textit{N-ras} codon 12.

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**Fig 2.** Detection of \textit{K-ras} mutations in two of 12 patients. (A) \textit{K-ras} codon 12 probe specific for ASP; (B) \textit{K-ras} codon 12 probe specific for GLY (wt); (C) \textit{K-ras} codon 13 probe with ASP; (D) \textit{K-ras} codon 13 probed with GLY (wt). Patient no. 4 shows an ASP mutation in \textit{K-ras} codon 12; patient no. 5 an ASP mutation in \textit{K-ras} codon 13.
Mutations in the ras proto-oncogenes are a major molecular event in AML and MDS in adults.\(^{1,3,8,16,21,23-25}\) Janssen et al previously found a mutation in the K-ras proto-oncogene in one patient with juvenile CML.\(^{20}\) Our data extend this observation to an unselected series of patients with a well-defined childhood MPS. Although BMT has been used successfully to treat childhood monosomy 7, there are little data regarding the risk of relapse.\(^{18,20}\) The observation that both children with ras mutations who underwent BMT suffered relapses is intriguing. Further investigation is indicated to assess the prognostic importance of ras mutations in monosomy 7 and other types of childhood MPS.

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