Patterns of Hematopoietic Lineage Involvement in Children With Neurofibromatosis Type 1 and Malignant Myeloid Disorders

By Darryl K. Miles, Melvin H. Freedman, Karen Stephens, Maria Pallavicini, Eric L. Sievers, Molly Weaver, Tom Grunberger, Patricia Thompson, and Kevin M. Shannon

Children with neurofibromatosis type 1 (NF1) are at increased risk of developing malignant myeloid disorders, particularly juvenile chronic myelogenous leukemia/juvenile myelomonocytic leukemia (JCML/JMML). We investigated bone marrows from 11 such patients (8 boys and 3 girls) and detected allelic losses at the NF1 locus in 4 of them and probable losses in 2 others. To determine which hematopoietic cell lineages were derived from the abnormal clones, Epstein-Barr virus (EBV)-transformed cell lines and CD34+ cells were analyzed from 3 children with JCML with allelic losses in unFractionated marrow. CD34 cells from these 3 patients lacked the normal NF1 allele, whereas EBV cell lines retained it. Erythroblasts plucked from the burst-forming unit-erythroid colonies of one of these children lacked the normal NF1 allele. We also studied a 10-month-old boy with NF1 who developed an unusual myeloproliferative syndrome. His bone marrow and EBV cell line both showed loss of the normal NF1 allele. In our series and in the literature, male sex and maternal transmission of NF1 were associated with the highest risk of myeloid leukemia. These data (1) provide strong genetic evidence that NF1 functions as a tumor-suppressor in early myelopoiesis, (2) confirm the clonal nature of JCML/JMML, (3) suggest that the elevation in fetal hemoglobin seen in JCML/JMML is a result of primary involvement of erythroid progenitors in the malignant clone, (4) show consistent loss of NF1 in the CD34 cells of affected children and show that the malignant clone may also give rise to pre-B cells in some cases, and (5) implicate epigenetic factors in the development of leukemia in children with NF1.

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NF1 is an autosomal dominant disorder with an incidence of 1 in 4,000.1

Affected individuals show pigmented abnormalities and are predisposed to benign and malignant neoplasms that typically arise in cells derived from the embryonic neural crest.1 Children (but not adults) with NF1 are at markedly increased risk of developing malignant myeloid disorders, including juvenile chronic myelogenous leukemia/juvenile myelomonocytic leukemia (JCML/JMML), bone marrow monosomy 7 syndrome (Mo7), and acute myelogenous leukemia (AML).2,3 The NF1 gene (NF1) was cloned in 1990,4 and encodes a protein called neurofibromin that binds to members of the p21 family (Ras) of signaling proteins and regulates their biochemical activity by accelerating hydrolysis of Ras-guanine nucleotide triphosphate (Ras-GTP) to Ras-guanine nucleotide diphosphate (Ras-GDP).5,6 A large body of experimental evidence implicates Ras proteins in normal and abnormal hematopoietic cell growth.7,8,12 In particular, proliferative responses to many hematopoietic growth factors are associated with increased intracellular levels of Ras-GTP15; activating Ras mutations are common in preleukemia and AML13,16; and mutant Ras alleles efficiently transform myeloid cell lines.17,18 Activating Ras mutations are found in the marrows of 20% to 40% of children with JCML, Mo7, or AML,19,20 but have not been detected in children with NF1.21 Furthermore, the normal NF1 allele was absent in bone marrow cells from 5 of 11 children with NF1 who developed myeloid disorders.22 Recently, biochemical and cell culture studies of leukemic bone marrow cells from children with NF1 and of hematopoietic cells harvested from embryos with homozygous inactivation of the murine NF1 gene (Nfi) have shown elevated levels of Ras-GTP, a decrease in the ability to accelerate GTP hydrolysis on Ras, activation of mitogen-activated protein (MAP) kinase (a downstream effector of Ras), and in vitro hypersensitivity to granulocyte-macrophage colony-stimulating factor (GM-CSF).24,25 Taken together, these results support the hypothesis that NF1 functions as a tumor-suppressor gene in immature myeloid cells and that neurofibromin negatively regulates hematopoietic growth through its effect on Ras-GTP.

Although the normal NF1 allele was absent in leukemic bone marrow cells of a few children with NF1,23 there are no data that address which hematopoietic lineages were involved in these malignant clones. In this study, we examined DNA extracted from subpopulations of bone marrow cells from a group of children with NF1 and malignant myeloid disorders for allelic loss at NF1. Of 11 samples tested, 4 showed losses and 2 others had probable NF1 gene deletions. Investigation of Epstein-Barr virus (EBV)-transformed cell lines, CD34+ bone marrow cells, and erythroblasts plucked from burst-forming unit-erythroid (BFU-E) colonies from some of the patients with allelic losses provided evidence that malignant transformation in JCML occurs in an immature progenitor
that is committed to myeloid differentiation. However, we surprisingly found loss of the normal NF1 allele in both EBV-transformed and unfractonated bone marrow cells from 1 child with an atypical clinical presentation. These new cases also extend previous observations that boys who inherit NF1 from their mothers are at the highest risk of developing myeloid disorders.

MATERIALS AND METHODS

Patient population and diagnostic criteria. The 11 patients included in this study were referred for study by pediatric oncologists throughout the world between 1992 and 1995. The experimental procedures were approved by the Institutional Review Board of the University of California, San Francisco. An International Working Group has suggested that JCM be renamed JMML because the latter provides a more accurate description of the underlying malignant myeloid disorder and eliminates potential confusion with Philadelphia chromosome-positive chronic myelogenous leukemia (CML; RR Castelberry et al, personal communication, April 1996). Accordingly, the term JMML is used in the succeeding sections of this report. Children diagnosed with JMML were diagnosed by pediatric hematologists at the referring institutions using a combination of clinical and laboratory criteria including (1) leukocytosis with an elevated absolute monocyte count, (2) the presence of immature myeloid precursors in the peripheral blood, (3) less than 30% blasts in bone marrow aspirates, and (4) the absence of the Philadelphia chromosome. Most of the patients had associated findings, including hepatosplenomegaly, thrombocytopenia, an elevated percentage of fetal hemoglobin for age, and/or in vitro hypersensitivity of colony-forming units–granulocyte-macrophage to granulocyte-macrophage colony-stimulating factor.

Polymorphic markers in the NF1 region. Investigating these leukemic samples for allelic loss was complicated by the fact that normal nonhematopoietic tissues from the same patients were not available to determine if their bone marrow showed loss of constitutional heterozygosity (LOH) in the NF1 region. We therefore extracted parental DNA and studied it in parallel with DNA from the patient bone marrows and, in some cases, from EBV-transformed lymphoblastoid cell lines. We routinely used 3 sequence polymorphisms within NF1 and 1 marker located centromeric of the gene to screen for allelic loss. The intragenic markers are EVI-20, an Alu repeat described by Xu et al, and a complex repeat described by Andersen et al; the flanking marker is UT-172. Some patient bone marrows showed a single allele at all 4 loci, but allelic loss could not be ascertained with certainty because DNA from one or both parents was not available. In these cases, we also investigated two new intragenic repeat polymorphisms described by Lazaro et al. All of the polymorphic loci were scored by amplifying DNA segments that contain a variable number of short nucleotide repeats with flanking oligonucleotide primers. DNA samples were amplified in a DNA Thermocycle Machine (Perkin-Elmer Applied Biosystems Inc, Foster City, CA). Polymerase chain reaction (PCR) was performed in reaction mixtures that include 10 pmol/L of respective 3' and 5' primers, 100 ng of target genomic DNA, 1 U of Taq polymerase (Perkin-Elmer Applied Biosystems Inc), and 100 mmol/L final concentrations of deoxynucleotides in a final reaction volume of 25 µL. 

Preparation of EBV-transformed lymphoblastoid cell lines. In most patients, mononuclear cells were isolated from patient peripheral blood samples by ficoll-histopaque (specific gravity, 1.077) density gradient centrifugation. The cells were washed three times in calcium and magnesium-free phosphate-buffered saline (CMF/PBS) and resuspended in 1 mL of RPMI 1640 medium supplemented with 20% fetal bovine serum, L-glutamine, and antibiotics. The number of viable lymphocytes was then determined by trypan blue exclusion. The cells were then resuspended in 4 mL of CMF/PBS supplemented with 0.5% human gamma globulin and transferred to T25 tissue culture flasks coated with anti-CD3 antibody (Applied Immune Sciences Inc, Santa Clara, CA) that had been prepared by washing four times with CMF/PBS. Mononuclear cells were incubated in the T25 anti-CD3 flasks for 1 hour at room temperature on a level surface without agitation. The nonadherent cells were then recovered by gently rocking the flask and removing the cell suspension. The cells were washed and resuspended at 1 × 10⁶ cells/mL. A total of 0.1 mL of EBV supernatant from cell line B95-8 was added per 1 × 10⁶ cells with thorough mixing. Cells were cultured at 1 to 2 × 10⁶ cells per well in a 24-well tissue culture plate. After 5 days, 1 mL of fresh media was added to each well. The cultures were fed twice weekly with 20% fetal bovine serum in RPMI 1640 supplemented with 25 mmol/L HEPES, L-glutamine, and antibiotics. Transformation was ascertained by the appearance of tightly formed cell clusters and usually occurred within 3 to 6 weeks. The EBV cell line from the patient shown in Fig 2 was prepared using a modification of the method of Netzel (the exact protocol is available from K. Stephens, University of Washington, Seattle).

Isolation of CD34+ cells. Frozen cells were thawed gradually and washed once in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum prewarmed to 37°C. Nucleated cells were labeled with 7.5 µmol/L Hoechst 33342 (HO) at 37°C for 60 minutes, washed in Hank's balanced salt solution containing 0.02% azide, and incubated at 4°C for 45 minutes with fluorescein isothiocyanate (FITC)-conjugated anti-CD34 antibody (Becton Dickinson [BD], San Jose CA). After antibody labeling, the cells were washed once with PBS. Dead cells were stained with 1 µg/mL propidium iodide (PI; Calbiochem, Santa Clara, CA) for 5 minutes at 20°C. Flow cytometric analysis was performed using a FACStar PLUS (BD) equipped with 2 argon lasers tuned to 488 nm and 351 to 364 nm (UV wavelength). Forward light scatter, perpendicular light scatter, and fluorescence signals were measured for each cell. Cell doublets and aggregates were excluded using forward light scatter processing. HO and PI fluorescence emissions (UV excitation) were collected through a 425-nm (±50-nm) bandpass and a 620-nm long pass filter, respectively. PI+/HO-positive events were considered to be viable nucleated cells. FITC emission (488 nm excitation) was collected through a 530-nm (±30-nm) bandpass filter. An isotype-matched antibody (Stimultest; BD) was used to define the immunofluorescence intensity above which cells were considered labeled. PI+/HO-positive CD34+ cells were sorted into a test tube containing RPMI medium and DNA was extracted as described previously. Two small numbers of bone marrow mononuclear cells available from these young children precluded using the cell sorter to purify additional hematopoietic subpopulations for DNA extraction and polymorphism analysis.

RESULTS

Clinical data on the 11 patients are presented in Table 1. The study group included 8 boys and 3 girls with a median age at disease onset of 22 ± 15 months. Eight children had JMML and 1 patient each had Mo 7, AML, or a myeloprolif-
Table 1. Features of Study Population

<table>
<thead>
<tr>
<th>Patient No. and Sex</th>
<th>Age at Onset</th>
<th>Diagnosis</th>
<th>Parent With NF1</th>
<th>Results With NF1 Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M</td>
<td>24 mo</td>
<td>Mo 7</td>
<td>Unknown</td>
<td>LOH</td>
</tr>
<tr>
<td>2M</td>
<td>6 mo</td>
<td>JMML</td>
<td>Neither</td>
<td>HZ</td>
</tr>
<tr>
<td>3M</td>
<td>14 mo</td>
<td>JMML</td>
<td>Mother</td>
<td>LOH</td>
</tr>
<tr>
<td>4M</td>
<td>6 mo</td>
<td>JMML</td>
<td>Mother</td>
<td>HZ</td>
</tr>
<tr>
<td>5M</td>
<td>22 mo</td>
<td>AMMoL</td>
<td>Neither</td>
<td>HZ</td>
</tr>
<tr>
<td>6M</td>
<td>30 mo</td>
<td>JMML</td>
<td>Mother</td>
<td>Probable LOH</td>
</tr>
<tr>
<td>7M</td>
<td>5 yr</td>
<td>JMML</td>
<td>Mother</td>
<td>LOH</td>
</tr>
<tr>
<td>8F</td>
<td>30 mo</td>
<td>JMML</td>
<td>Mother</td>
<td>LOH</td>
</tr>
<tr>
<td>9M</td>
<td>19 mo</td>
<td>MPS</td>
<td>Mother</td>
<td>HZ</td>
</tr>
<tr>
<td>10F</td>
<td>12 mo</td>
<td>JMML</td>
<td>Neither</td>
<td>HZ</td>
</tr>
<tr>
<td>11F</td>
<td>18 mo</td>
<td>JMML</td>
<td>Unknown</td>
<td>Probable LOH</td>
</tr>
</tbody>
</table>

Abbreviations: HZ, retains heterozygosity; AMMoL, acute myelomonocytic leukemia.

CD34+ cells from the frozen bone marrow mononuclear cells of patients no. 3, 7, and 8; and plucked erythroblasts from BFU-E colonies grown from the bone marrow of patient no. 3. As shown in Fig 1, the EBV cell lines of these 3 patients retained both NF1 alleles, whereas the CD34+ cells consistently showed LOH. DNA extracted from the BFU-E colonies of patient no. 3 also showed LOH. Because patients no. 3, 7, and 8 had JMML, these data provide evidence that leukemic transformation in this disorder occurs at the level of an immature progenitor that is committed to myeloid differentiation and shows involvement of erythroid progenitors in the abnormal clone.

An EBV line was also available from a child with an MPS reported previously (patient no. 8 in Shannon et al23). In contrast to the 3 JMML patients, we detected absence of the normal maternal NF1 allele and of flanking polymorphic markers in both his EBV line and bone marrow (Fig 2). This 10-month-old boy had an unusual myeloproliferative disorder characterized by recurrent fevers, generalized lymphadenopathy, hepatosplenomegaly, and numerous cafe au lait spots. His peripheral white blood cell count was 94,000/μL, consisting predominately of myeloid cells in various stages of differentiation. A bone marrow aspirate was hypercellular, with a myeloid to erythroid ratio of 20:1. Myeloblasts constituted 5% of the nucleated cells. Cyogenetic analysis of unstimulated bone marrow was normal and the level of fetal hemoglobin was normal for age. Aside from the fact that his marrow did not express BCR-ABL fusion transcripts, his presentation was indistinguishable from Philadelphia chromosome-positive CML.

Our series included 5 boys and 1 girl with familial NF1, all of whom inherited the disease from their mothers (Table 1). When these cases are combined with the published data summarized in Table 2, skewing is evident with respect to the sex of the parent transmitting NF1 as well as the sex of the affected child. The ratios of maternal to paternal transmission and of boys to girls with myeloid disorders were 2.3:1 and 4.5:1, respectively. The most pronounced difference were seen between boys who inherited NF1 from their mothers (18 cases) and girls who inherited it from their fathers (1 case). Using χ2 analysis, there was a significantly greater proportion of mothers transmitting NF1 to children who developed leukemia (P < .02) and a significantly greater proportion of affected boys with familial NF1 (P < .01).
Fig 2. Analysis of bone marrow and EBV cell line DNA from a child who developed an unusual MPS with the polymorphic markers UT172 (lanes 1 through 5) and D17S787 (lanes 6 through 9). Different alleles are designated in order of size, beginning with the letter “A.” DNA from the following sources were amplified and resolved: lanes 1 and 6, mother; lanes 2 and 7, father; lane 3, patient blood; lanes 4 and 8, patient EBV cell line; lanes 5 and 9, unfractionated patient bone marrow. UT172 and D17S787 are located centromeric and telomeric of NF1, respectively. The EBV cell line and bone marrow also showed LOH with the intragenic NF1 marker described by Andersen et al.²

DISCUSSION

Although substantial progress has been made in characterizing the genes that are altered during leukemogenesis and in understanding how these somatic mutations perturb hematopoietic growth, most of this work has focused on dominant genetic changes such as translocations that activate putative proto-oncogenes. Much less is known about the role of tumor-suppressor gene alterations in leukemic transformation and progression. General features of tumor-suppressor genes include (1) that germline mutations are associated with an increased risk of specific cancers; (2) that the single normal allele is inactivated by deletion, gene conversion, or point mutation during tumorigenesis; and (3) that the proteins encoded by tumor-suppressor genes normally function to negatively regulate cell growth.²²,²³ NF1 fulfills all of these criteria in myeloid leukemia. First, children with NF1 are at a markedly increased risk of developing malignant myeloid disorders, particularly JMML.²²,²³ Second, when data from the patients included in this study are combined with cases in a previous report,²³ leukemic bone marrows from 9 of 18 children have definitely shown allelic losses and samples from 2 other patients have probable deletions. Similarly, mice that are heterozygous for a targeted disruption of NF1 are predisposed to myeloid leukemia, and these bone marrows delete the wild-type allele.²³,²⁴ When LOH has been detected in leukemic cells from children with familial NF1, the NF1 allele inherited from the unaffected parent has been deleted in every case studied to date. It is likely that the bone marrows that retained both copies of NF1 sustained subtle alterations such as point mutations in the normal allele that caused loss of function without allelic loss. Third, the identification of neurofibromin as a GAP for Ras, the conspicuous absence of RAS mutations in the leukemic bone marrows of children with NF1²² and direct evidence that the Ras signaling pathway is activated in leukemic cells from children with NF1 and in murine NF1−/− hematopoietic cells²²,²³ provide strong evidence that neurofibromin normally restrains the growth of myeloid cells by negatively regulating Ras.

JMML is the most common leukemia that arises in children with studies of rare patients with a marker cytogenetic alteration in the bone marrow³⁵ and, more recently, by showing a clonal pattern of hematopoiesis by the criteria of a nonrandom pattern of X chromosome inactivation in girls with JMML.³⁶ Loss of the normal allele of NF1 in some patients provides a third independent line of evidence that supports the clonal nature of JMML. The existence of this genetic marker of the leukemic clone allowed us to ascertain patterns of hematopoietic lineage involvement in 3 children with NF1 who developed JMML. We consistently found loss of the normal NF1 allele in unfractionated bone marrow samples and in CD34+ cells, but retention of the normal allele in cell lines derived from pre-B lymphocytes. Our data suggest that JMML is not a true stem cell disorder but that malignant transformation occurs in a hematopoietic cell that is restricted to myeloid differentiation. This is in contrast to Philadelphia-positive CML and a number of other malignant myeloid disorders in which involvement of both B lymphocytes and myeloid cells has been shown by a number of approaches.²⁷,²⁸ The myelodysplasias associated with monosomy 7 or del(5q) are similar to JMML in that the cytogenetic abnormality is apparently restricted to myeloid cells.³¹,³² The observation that CD34+ cells have deleted the normal parental NF1 allele is consistent with the aberrant pattern of myeloid progenitor colony growth in response to GM-CSF that is a laboratory feature of JMML.³³,³⁴ In addition, JMML bone marrows contain a higher percentage of CD34+ cells than normal samples (mean, 4.9%; range, 3.8% to 5.8% in JMML v mean, 1.8%; range, 0.6% to 2.9% in normal marrows; Freedman and Grunberger, unpublished data). These findings are consistent with data from mice reconstituted with NF1−/− deficient hematopoietic cells that develop a JMML-like syndrome that is associated with a marked increase in the percentage of immature Mac-1+, Gr-1+ myeloid progenitor cells in the marrow.³³ Although all of these data show a myeloid/erythroid pattern of involvement in JMML, it is possible that this disorder arises from

Table 2. Parental Transmission of Familial NF1 in Children With Myeloid Disorders

<table>
<thead>
<tr>
<th>Sex of Affected Child</th>
<th>Sex of Parent With NF1</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Mother</td>
<td>Father</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>19</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>4</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

Includes the patients in this study; cases described previously as reviewed in Shannon et al.³ and cases in Shannon et al.²³
a more primitive cell that only achieves clonal dominance in these lineages.

JMML is characterized by elevated levels of fetal hemoglobin in vivo and by qualitatively abnormal growth of BFU-E colonies in vitro. A previous report that described a marker cytogenetic abnormality in the BFU-E colonies of a child with JMML suggested that the erythroid lineage is derived, at least in part, from differentiation of the malignant clone. Papayannopoulou et al observed an embryonic pattern of γ- and ε-globin synthesis in JMML erythroid colonies and more recent work has shown constitutive expression of the erythroid-lineage genes GATA-1, EPOR, α-globin, and γ-globin in myeloid progenitor colonies grown from JMML patients. These qualitative abnormalities of in vitro hematopoiesis are consistent with the idea that the disordered pattern of erythropoiesis is an intrinsic characteristic of progenitors derived from the malignant clone. Our data showing loss of the normal NFI allele in erythroblasts harvested from the BFU-E colonies of a JMML patient confirm this hypothesis. Although EBV-transformed cells from patients with JMML consistently retained both NFI alleles, we observed allelic loss in both unFractionated bone marrow and EBV-transformed cells from a child with an atypical MPS. This case shows that loss of NFI can occur in an immature hematopoietic precursor that is capable of giving rise to both lymphoid and myeloid progeny. It is intriguing that the clinical course of this child was suggestive of Philadelphia-positive CML, because CML is associated with activation of the Ras signaling pathway through binding of Bcr-Abl to the Grb2 adapter protein. Transfection of BCR-ABL into hematopoietic and nonhematopoietic cell lines results in phosphorylation of other proteins in the Ras signaling pathway, including p120GAP, MAP kinase, and Jun N-terminal kinase, and recent data implicate Ras activation as essential for BCR-ABL-induced transformation. Additional experiments are required to characterize the full spectrum of hematopoietic lineage involvement in the myeloid disorders associated with NFI and to ascertain whether the level at which transformation occurs correlates with clinical behavior.

Leukemic transformation is much less common in children with NFI than in patients who inherit germline mutations that predispose to retinoblastoma or Wilms' tumor. This suggests that loss of the normal NFI allele occurs very infrequently in hematopoietic cells or, alternately, that inactivation of NFI must cooperate with other somatic and inherited genetic alterations to produce full leukemic transformation. It is not yet possible to distinguish between these possibilities. On the one hand, the fact that mice transplanted with NFI-/y fetal liver cells consistently develop a JMML-like disorder shows that inactivation of NFI is sufficient to deregulate myeloid growth. However, two lines of evidence suggest that loss of NFI function interacts with other genetic factors in human leukemogenesis. First, some NFI patient bone marrows show cytogenetic abnormalities, particularly monosomy 7. Second, epidemiologic data implicate epigenetic factors in the development of myeloid disorders in children with NFI. According to the Knudson model, inheriting a germline NFI mutation should predispose to cancer in a manner that is independent of the sex of either the transmitting parent or the affected child. This is not true in children with NFI as boys who inherit NFI from their mothers are at especially high risk of developing myeloid disorders. Although the male predilection seen among children with NFI is similar to the distribution of cases of JMML and Mo 7 in the general population, it is striking that this ratio is retained even in the context of a strong inherited cancer predisposition. In addition, the fact that leukemia is much more common among children who inherit NFI from their mothers is unexpected and is not easily explained. The proposal that the maternal NFI allele might be imprinted in hematopoietic cells (reviewed in Half and Sapienza) is not supported by our finding that mutant NFI alleles of both maternal (6 cases) and paternal (3 cases) origin have been retained in patient bone marrows (Shannon et al and these data).

The results of this study firmly establish NFI as a tumor-suppressor gene in immature hematopoietic cells and raise a number of questions with respect to the role of NFI inactivation in leukemogenesis. The reason children with NFI are strongly predisposed to myeloid disorders whereas adults are not is unknown. It is interesting that the MPS that develops in mice that are heterozygous for a targeted deletions of the murine NFI gene have only been observed in adult animals. In addition, although we have shown that the CD34+ compartment in JMML bone marrows consists largely of cells derived from the malignant clone, it is not clear if transformation occurs at this level of differentiation or if a subpopulation of more primitive stem cells is also affected. Our data in the child with atypical MPS provide direct evidence that his malignant clone included some cells that are capable of both myeloid and lymphoid differentiation. Finally, the role of epigenetic factors in the pathogenesis of JMML and Mo 7 in children with and without NFI and the nature of the genetic events that cooperate in leukemogenesis requires further study.

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