

LOSS OF THE NORMAL NF1 ALLELE FROM THE BONE MARROW OF CHILDREN WITH TYPE 1 NEUROFIBROMATOSIS AND MALIGNANT MYELOID DISORDERS

KEVIN M. SHANNON, M.D., PETER O'CONNELL, PH.D., GEORGE A. MARTIN, B.A., DOROTHY PADERANGA, KRISTIN OLSON, B.A., PATRICIA DINNDORF, M.D., AND FRANK McCORMICK, PH.D.

Abstract *Background.* Children with type 1 neurofibromatosis (NF-1) are at increased risk for malignant myeloid disorders. Analysis of the NF-1 gene (*NF1*) suggests that the function of its product, neurofibromin, is reduced in affected persons and that *NF1* belongs to the tumor-suppressor class of recessive cancer genes. This model is consistent with evidence that neurofibromin accelerates the intrinsic guanosine triphosphate–hydrolyzing activity of the Ras family of regulatory proteins. Loss of constitutional heterozygosity has not been reported in the benign tumors associated with NF-1, however, and has only been detected in a few malignant neural-crest tumors and in some tumor-derived cell lines.

Methods. We studied DNA extracted from the bone

marrow of 11 children with NF-1 in whom malignant myeloid disorders developed and from parental leukocytes. We used a series of polymorphic markers within and near *NF1* to determine whether leukemogenesis was associated with structural alterations of the gene.

Results. Bone marrow samples from five patients showed loss of heterozygosity. In each case, the *NF1* allele was inherited from a parent with NF-1 and the normal allele was deleted.

Conclusions. These data provide evidence that *NF1* may function as a tumor-suppressor allele in malignant myeloid diseases in children with NF-1 and that neurofibromin is a regulator of Ras in early myelopoiesis. (N Engl J Med 1994;330:597-601.)

PROTEINS encoded by the *RAS* family of proto-oncogenes regulate cellular growth and differentiation by cycling between an active state in which they are bound to guanosine triphosphate (Ras–GTP) and an inactive state in which they are bound to guanosine diphosphate (Ras–GDP).^{1,2} During the development of cancer in humans, these genes commonly acquire activating point mutations that perturb the biochemical activity of Ras proteins by elevating the level of Ras–GTP.³ Yeast and mammalian GTPase-activating proteins normally regulate the biologic activity of Ras proteins by accelerating the hydrolysis of GTP. Because it is Ras–GTP that actively transduces signals, GTPase-activating proteins act (at least in part) as negative regulators of Ras function.

Neurofibromin, the protein encoded by the gene that is mutated in patients with the autosomal dominant genetic disorder neurofibromatosis type 1 (NF-1), shows sequence homology with yeast and mammalian GTPase-activating proteins.⁴⁻⁶ Moreover, the GTPase-activating protein domain of neurofibromin binds to Ras and accelerates the hydrolysis of GTP at physiologically relevant concentrations.^{4,7,8} Patients with NF-1 are at increased risk for certain benign and malignant neoplasms; these tumors primarily arise in cells derived from the embryonic neural crest.⁹ Taken

together, the strong association of activating *RAS* mutations with oncogenesis, the increased risk of certain malignant conditions in patients with NF-1, and the biochemical activity of neurofibromin on Ras proteins suggest that *NF1* belongs to the tumor-suppressor class of recessive cancer genes.¹⁰ This model predicts that acquired genetic alterations that inactivate the single normal *NF1* allele contribute to the formation of cancer in persons with NF-1. Recent data derived from structural and biochemical studies of malignant tumors removed from patients with NF-1 support the hypothesis that *NF1* functions as a tumor suppressor in neural-crest cells.¹¹⁻¹⁷

Children with NF-1 are predisposed to malignant myeloid diseases, particularly preleukemic myelodysplastic syndrome and myeloproliferative syndrome (MPS). These disorders are characterized by deregulated clonal proliferation of immature hematopoietic cells that show some myeloid differentiation in vivo.¹⁸ Juvenile chronic myelogenous leukemia and monosomy 7 syndrome of the bone marrow account for most cases of MPS in children. These two disorders share many features, including a similar age of onset, a tendency to affect boys, prominent enlargement of liver and spleen, leukocytosis, the absence of the Philadelphia chromosome, and a poor prognosis, with either progression to acute myelogenous leukemia (AML) or death from intercurrent problems.¹⁸ Although MPS is an uncommon complication of NF-1 in childhood, NF-1 constitutes as many as 10 percent of the spontaneous cases of MPS in children.¹⁸⁻²¹ The association between NF-1 and MPS in childhood is particularly intriguing because these malignant conditions typically appear early in life and affect a cell line not derived from neural-crest cells, and because oncogenic *RAS* mutations occur frequently in MPS and AML but not in neural-crest tumors.^{3,22} We have examined DNA samples from 11 families in which a malignant myeloid disorder developed in a child

From the Department of Pediatrics, University of California, San Francisco (K.M.S., D.P., K.O.); the Department of Pathology, University of Texas Health Science Center, San Antonio (P.O.); Onyx Pharmaceuticals, Richmond, Calif. (G.A.M., F.M.); and the Division of Pediatric Oncology, Children's National Medical Center, Washington, D.C. (P.D.). Address reprint requests to Dr. Shannon at the Department of Pediatrics, University of California, Rm. U-432, San Francisco, CA 94143-0724.

Supported in part by grants from the National Neurofibromatosis Foundation and the Children's Cancer Research Fund, by the U.S. Navy Clinical Investigation Center Program (protocols 90-48-2807 and 90-018), by an American Cancer Society Junior Faculty Research Award (JFRA-471, to Dr. Shannon), and by a grant from the National Cancer Institute to the Children's Cancer Group. The opinions and assertions expressed in this work are those of the authors and do not necessarily reflect the views of the Children's Cancer Research Fund or the Department of the Navy.

with NF-1 and present data implicating *NFI* as a tumor suppressor in hematopoietic cells of the myeloid lineage.

METHODS

We studied 10 boys and 1 girl with NF-1 and MPS (9 patients) or AML (2 patients) who were treated at pediatric referral centers. Parental DNA was used for comparison studies. The epidemiologic and clinical features of the patients are summarized in Table 1. Bone marrow samples from the four patients with monosomy 7 (Patients 3, 4, 5, and 6) were studied previously with probes linked to *NFI*, and none showed loss of heterozygosity.²¹ We examined bone marrow samples from all children with NF-1 and malignant myeloid disorders for whom parental DNA was available for comparison studies. The patients were referred by pediatric oncologists throughout North America between 1989 and 1993. The experimental procedures were approved by the institutional review board of the University of California, San Francisco, and informed consent was obtained from the families who participated.

We prepared DNA from peripheral-blood samples from the parents and from bone marrow from the patients using standard methods, as described elsewhere.^{21,23} Southern blotting and hybridization with complementary DNA probes were performed as previously described,^{21,23} except that we transferred digested DNA samples from agarose gels to nylon membranes (Hybond N+ membranes, Amersham) under alkaline conditions according to the manufacturer's instructions. The membranes were rinsed twice in 2× saline sodium citrate buffer (0.3 M sodium chloride and 0.03 M sodium citrate) before they were hybridized.

We examined five sequence polymorphisms within *NFI*: probes AE25²⁴ and EVI-2B²⁵ identify restriction-fragment-length polymorphisms on Southern blots, whereas EVI-20 and the markers described by Xu and associates²⁶ and Andersen et al.²⁷ consist of variable numbers of short nucleotide repeats and are detected by oligonucleotide-directed amplification with the polymerase chain reaction (PCR), followed by gel electrophoresis. A sixth marker, UT172, is a polymorphic Alu repeat located approximately 1.5 Mb centromeric of *NFI*. We also used two markers from the short arm of chromosome 17 to examine blood and marrow samples from these families: probe YNZ22 is located near the p53 tumor-suppressor gene,²⁸ and a pair of oligonucleotide primers detect an intragenic p53 polymorphism by PCR.²⁹ The investigation of these samples for loss of heterozygosity was complicated by the fact that very little DNA was available from some patients and by the fact that we had no source of normal tissues in most cases and therefore assessed loss of heterozygosity by comparing parental DNA with patient DNA obtained from bone marrow.

DNA samples were amplified in a DNA Thermocycle machine (Perkin-Elmer Cetus). We performed PCR in reaction mixtures that included 10 pM each of 3' and 5' primers, 100 ng of target genomic DNA, 1 unit of *Taq* polymerase (AmpliTaq, Cetus), and

100 μM (final concentration) of deoxynucleotides in a final reaction volume of 50 μl. We incorporated [³³P]deoxy-ATP into the DNA fragments generated in the PCR procedure by adding 2 μl (10 μCi) of [³³P]deoxy-ATP per 500 μl of the reaction mixtures and by decreasing the concentrations of unlabeled deoxy-ATP to 50 mM. In some experiments, we end-labeled the 5' oligonucleotide primer with [³²P]*y*-ATP before performing PCR instead of incorporating [³³P]deoxy-ATP during the amplification process. Labeled PCR products were separated on sequencing gels (measuring 0.4 mm by 20 mm by 60 mm) run at 60 to 80 W of constant power for two to four hours. The gels were placed in plastic wrap and exposed to x-ray film for one to five days at room temperature.

The EVI-20 polymorphism was detected with forward primer 5'-CCCATACCTAGTTCTAAAGTCTGT3' and reverse primer 5'-TAACAATTGTGGAACACTGCAGCAATTATT3'. Amplification consisted of 26 cycles of denaturation at 94°C for one minute, annealing at 67°C for one minute, and extension at 72°C for one minute; the magnesium chloride concentration was 2 mM, and the alleles were detected on a gel containing 5 percent acrylamide, 6 M urea, and 32 percent deionized formamide. We tested the amplification products on a 5 percent acrylamide minigel (measuring 1.0 mm by 10 cm by 10 cm) before loading the large gels and performed four to eight additional cycles of PCR if the products were not visualized. This procedure minimized the amplification of background products. EVI-20 detects four common alleles ranging from 200 to 210 base pairs (bp). The UT172 polymorphism was detected with forward primer 5'-GGTGAAAGAGCAAGACTCTGTCAC3' and reverse primer 5'-CCCCTTGATTGTAAGCNACAGAAC3'. Amplification consisted of 32 cycles of denaturation at 94°C for 45 seconds, annealing at 52°C for 45 seconds, and extension at 72°C for 45 seconds; the magnesium chloride concentration was 2.5 mM, and the alleles were detected on a gel containing 6 percent acrylamide, 6 M urea, and 10 percent deionized formamide. UT172 detects four common alleles ranging from 100 to 120 bp. To detect the other loci, we used amplification techniques described by Xu et al.,²⁶ Andersen et al.,²⁷ and Futreal et al.,²⁹ as recommended by the authors.

RESULTS

Analysis of DNA from all 11 families was informative at one or more polymorphic sites within *NFI*. Analysis of bone marrow showed that heterozygosity was retained in six patients (Patients 3, 4, 5, 7, 9, and 10) and that a parental *NFI* allele was lost in five others (Patients 1, 2, 6, 8, and 11 in Table 1) (Fig. 1). All five children with loss of heterozygosity had familial NF-1, and in each case the bone marrow retained the *NFI* allele inherited from the affected parent. The bone marrow of Patient 1 had loss of heterozygosity at all five intragenic loci (Fig. 1A). The absence of the normal paternal AE25 fragment in the bone marrow from this patient with juvenile chronic myelogenous leukemia directly demonstrates a structural deletion affecting the *NFI* coding region. Two of the five bone marrow samples showing loss of heterozygosity within *NFI* (Patients 6 and 8) also showed loss of heterozygosity at UT172; analysis of the other three samples with this marker was uninformative.

The p53 tumor-suppressor gene, located on the short arm of chromosome 17, is the most common target for loss-of-function mutations in human cancers.³⁰ *NFI* is also on chromosome 17, at band q11.2. We

Table 1. Features of Children with NF-1 and Malignant Myeloid Disorders.*

PATIENT NO.	SEX	AGE AT ONSET	DISORDER	PARENT WITH <i>NFI</i>	RESULTS WITH <i>NFI</i> MARKERS	RESULTS WITH p53 MARKERS
1	M	10 mo	JCML	Mother	LOH	HZ
2	M	10 mo	AML	Father	LOH	HZ
3	M	4 yr	Mo 7	Neither	HZ	NI
4	M	7 yr	AML†	Mother	HZ	HZ
5	M	9 mo	Mo 7	Mother	HZ	HZ
6	M	7 mo	Mo 7	Mother	LOH	HZ
7	M	3 yr	JCML	Neither	HZ	HZ
8	M	9 mo	CMM ^L	Father	LOH	HZ
9	M	2 yr	JCML	Father	HZ	NI
10	M	4 yr	JCML	Mother	HZ	HZ
11	F	18 mo	JCML	Father	LOH	HZ

*JCML denotes juvenile chronic myelogenous leukemia, LOH loss of heterozygosity, HZ heterozygous, Mo 7 MPS with monosomy, NI not informative, and CMM^L chronic myelomonocytic leukemia.

†Patient 4 had AML with monosomy 7 in the leukemic clone.

therefore examined bone marrow samples from the patients with probe YNZ22²⁸ (located near p53) and at a polymorphic site within p53.²⁹ As shown in Table 1, analysis of 9 of 11 bone marrow samples (including

all 5 showing loss of heterozygosity at *NFI*) with one of these markers was informative, and all demonstrated heterozygosity.

These results in patients with NF-1 suggested that

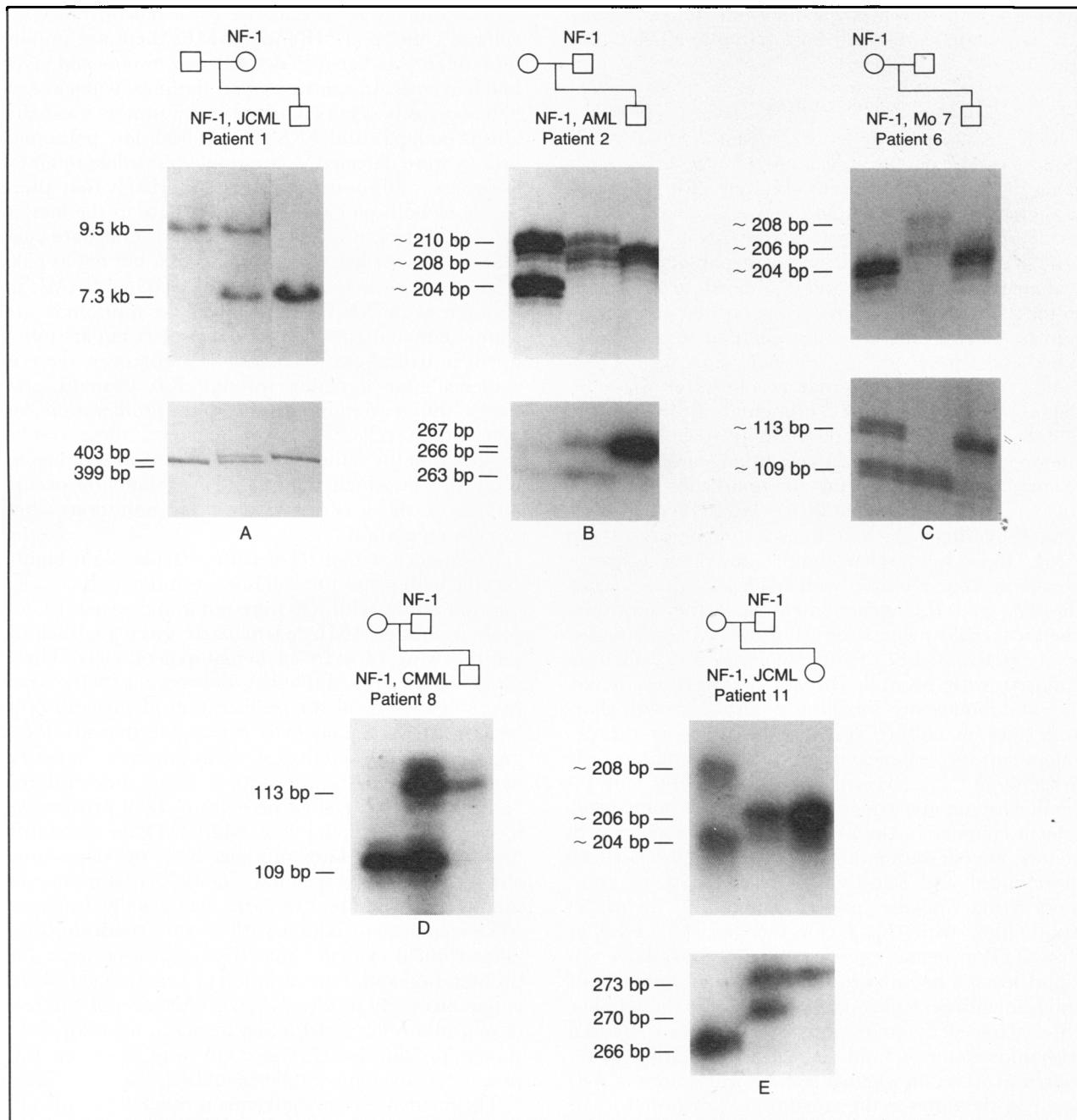


Figure 1. Loss of Heterozygosity within and near *NFI* in Bone Marrow Samples from the Patients.

In Panel A, DNA extracted from a bone marrow sample from Patient 1 and from a blood sample from his parents was digested with EcoRI, hybridized with probe EVI-2B (top blot), and amplified with primers that detect a polymorphic *NFI* Alu repeat²⁶ (bottom blot). The patient's DNA shows only the mutant maternal *NFI* gene. In Panel B, DNA from Patient 2 and his parents was amplified with primers that detect the EVI-20 repeat (top blot) and the sequence polymorphism described by Andersen et al.²⁷ (bottom blot). In the patient's DNA, the normal maternal *NFI* gene is lost and the mutant paternal allele is retained. In Panel C, DNA from Patient 6 and his parents was amplified with primers that detect the EVI-20 (top blot) and UT172 (bottom blot) polymorphisms. The mutant maternal *NFI* allele is retained in this patient with monosomy 7 (Mo 7) of the bone marrow. In Panel D, DNA from Patient 8 and his parents was amplified with primers that detect the UT172 polymorphism. The normal maternal allele is present in the bone marrow of Patient 8. In Panel E, DNA from Patient 11 and her parents was amplified with primers that detect the EVI-20 polymorphism (top blot) and the polymorphism described by Andersen et al.²⁷ (bottom blot). The maternal *NFI* allele is deleted in the patient. JCML denotes juvenile chronic myelogenous leukemia, and CMML chronic myelomonocytic leukemia.

NF1 might be a target for acquired mutations in children with MPS and AML who do not have NF-1. To address this question, we used the four *NF1* polymorphisms that can be detected by PCR to study 27 consecutive children with MPS and monosomy 7. Analysis of 25 of the samples was informative. All 25 bone marrow samples retained both parental alleles (data not shown).

DISCUSSION

Our results provide evidence that *NF1* acts as a tumor suppressor in myeloid cells *in vivo*, since bone marrow samples from children with NF-1 and malignant myeloid disorders showed a high frequency of loss of heterozygosity at *NF1*, consistently retained the mutant *NF1* allele of a parent with clinical neurofibromatosis, and remained heterozygous in the p53 region. These data suggest that neurofibromin is essential for growth regulation in a cell line not derived from neural-crest cells. Our results provide a coherent model that accounts for three independent clinical and experimental observations in childhood MPS. First, data implicating *NF1* as a tumor-suppressor allele in the pathogenesis of these leukemias readily explain the markedly increased incidence of MPS in children with NF-1.¹⁸⁻²¹ Second, activating *RAS* mutations are common in both AML and adult preleukemia^{3,22} and have been reported in some children with MPS.^{31,32} Mutations of the *NF1* and *RAS* genes might have the same biochemical consequences for cell growth (i.e., increased levels of Ras-GTP). Third, bone marrow cells from children with juvenile chronic myelogenous leukemia and monosomy 7 exhibit abnormal growth characteristics in culture systems that support the development of colonies derived from hematopoietic progenitors.³³⁻³⁵ Cells obtained from patients with juvenile chronic myelogenous leukemia and monosomy 7 form colonies in the absence of exogenous growth factors. Recent data indicate that these cells also have pronounced and selective hypersensitivity to granulocyte-macrophage colony-stimulating factor.^{34,35} Stimulation with this factor increases the level of Ras-GTP in hematopoietic cell lines.³⁶ We have not found loss of heterozygosity in the bone marrow of children with preleukemia and AML who do not have NF-1. However, our data are restricted to children with monosomy 7, and further investigation is required to ascertain whether somatic alterations of *NF1* occur in the other malignant myeloid disorders.

Investigation of neurofibrosarcomas and neurofibrosarcoma cell lines derived from patients with NF-1 has shown loss of constitutional heterozygosity, decreased *in vitro* activity of GTPase-activating proteins, and elevated levels of Ras-GTP.^{11-13,15,16} Legius et al.¹⁷ identified a 200-kb deletion of *NF1* in a fibrosarcoma with loss of heterozygosity at all chromosome 17 alleles tested. Loss of heterozygosity with retention of the mutant *NF1* allele has also been reported in pheochromocytomas from a few patients with NF-1.¹⁴

Finally, recent data indicating that cultured neuroblastoma³⁷ and melanoma³⁸ cell lines frequently show homozygous inactivation of *NF1* provide further evidence that loss of neurofibromin function confers a growth advantage in cells derived from the neural crest. Although these data are consistent with our results in childhood MPS and AML, there are important differences between neural-crest tumors and myeloid leukemia. In contrast to our findings, when loss of heterozygosity occurs in neural-crest tumors it usually affects both p53 and *NF1*.^{12,13,17} In addition, point mutations were detected in the single p53 allele retained in two neurofibrosarcomas,¹² and it is likely that alterations of both p53 and *NF1* contribute to the loss of growth control in neurofibrosarcomas. Our data suggest that loss of heterozygosity at *NF1*, but not at p53, is involved in the pathogenesis of MPS and AML in children with NF-1. Activating *RAS* mutations are common in malignant myeloid disorders but are infrequent in neural-crest tumors.^{3,22,32} Moreover, there is evidence that signaling through Ras proteins promotes differentiation, rather than proliferation, of neural-crest cells.³⁹⁻⁴¹ Taken together, these results suggest that the cellular context (neural crest or hematopoietic) in which the loss of *NF1* function occurs influences the way in which these mutations alter growth regulation.

The association of NF-1 with preleukemia is highly specific with respect to cell lineage and age. It is striking that adults with NF-1 are not at increased risk for preleukemia or AML, particularly given the lifelong self-renewing capacity of hematopoietic cells. These observations suggest that neurofibromin activity is not required to control the proliferation of myeloid cells beyond early childhood. It is possible that a critical growth-limiting activity of neurofibromin could be overcome by maturation to a more differentiated "adult type" of myeloid progenitor, by a progressive increase in the activity of p120 GTPase-activating protein or other related proteins, or by developmental changes in the extracellular signals that stimulate or inhibit growth. The dramatic and usually transient MPS seen in some infants with Down's syndrome provides clinical evidence that the balance between the proliferation and differentiation of hematopoietic cells is tenuous early in life.⁴² Characterization of the role of neurofibromin in fetal and neonatal hematopoiesis should provide insights into the way in which this process is developmentally regulated.

The incidence of preleukemia is much lower in children with NF-1 than is the risk of cancer in patients who carry germ-line mutations that predispose them to Wilms' tumor and retinoblastoma.¹⁰ It is possible that the loss of the normal *NF1* allele is necessary but not sufficient for leukemic transformation in children with NF-1. The presence of nonrandom cytogenetic abnormalities such as monosomy 7 in the bone marrow of some children with NF-1 and MPS provides evidence that leukemogenesis is a multistep process. In addition, epidemiologic data implicate epigenetic

mechanisms in the pathogenesis of these disorders. Childhood MPS shows a strong male preponderance, particularly during the first year of life. We observed loss of heterozygosity at *NF1* in the only girl in our series. This finding suggests that the mechanism by which germ-line *NF1* mutations predispose children to leukemia is the same in boys and girls, although the risk is much higher in boys. Our finding that either mutant maternal or paternal NF-1 alleles were retained in the bone marrow of children with familial NF-1 provides evidence that *NF1* is not imprinted in hematopoietic cells.

Germ-line *NF1* mutations are associated with a variety of malignant neoplasms of neural-crest origin. This observation and biochemical data from studies of cultured cell lines derived from tumor cells implicate neurofibromin as a major regulator of Ras proteins in these tissues. Our data suggest that neurofibromin has an important role in controlling the growth of myeloid cells and suggest that hyperactive Ras proteins contribute to the abnormal cellular proliferation seen in childhood MPS.

We are indebted to Dr. Y.W. Kan for ongoing advice and support; to Drs. Irwin Bernstein and Franklin Smith, who direct the Children's Cancer Group Acute Myelogenous Leukemia Reference Laboratory; to Drs. Beverly J. Lange and Greg Thomas for encouragement and samples from patients; to Dr. Robert Weiss for the primer sequences for EVI-20; to Dr. Ray White for the primer sequences for UT172; to Susan Tarle, Dr. Francis Collins, and Dr. Lone Andersen for oligonucleotide primer sequences and for their gift of probes AE25 and EVI-2B; to Drs. Gideon Bollag, Simon Cook, and Marc Hansen for helpful conversations; to Drs. Sarah Chaffee, L.C. Chan, Peter Emanuel, Steve Feig, Carolyn Felix, Jack Kelleher, Margaret Masterson, Jack Priest, Narayan Shah, Eric Sievers, Peter Steinherz, Brian Wickland, and Jan Watterson for providing samples from patients; and to the families who participated.

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