Title

Allelic loss at the NF1 locus and lineage involvement in children with type 1 neurofibromatosis (NF1) and malignant myeloid disorders

Author

Miles, Darryl,

Publication Date

1996

Peer reviewed|Thesis/dissertation

Allelic Loss at the *NF1* Locus and Lineage Involvement in Children with Type 1 Neurofibromatosis (NF1) and Malignant Myeloid Disorders

by

Darryl Miles

A Thesis

Submitted in partial satisfaction of the
requirements for the M.D. with Thesis Program
of the
University of California, San Francisco

Table of Contents

III Results

IV Discussion

V References 32-39

List of Figures

List of Tables

Abstract

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 two others. Epstein-Barr virus (EBV)-transformed cell lines and $CD34^+$ stem cells were analyzed from 3 children with JCML/JMML. $CD34^+$ cells from these 3 patients lacked the normal *NF1* allele, whereas EBV cell lines retained it. Erythroblasts plucked from the burst forming unit-erythroid (BFU-E) colonies of one of these children also lacked the normal *NF1* allele. We also studied a 10-month old boy with NF1 with an unusual myeloproliferative syndrome whose bone marrow and EBV cell line both showed loss of the normal *NF1* allele. In our series and in the literature, male gender and maternal transmission of NF1 were associated with the highest risk of myeloid leukemia. Theses 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 elevated levels of fetal hemoglobin seen in JCML/JMML is a result of primary involvement of the erythroid progenitors in the malignant clone, and (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 leukemias in children with NF1.

Acknowledgments

I would like to thank the following people for their invaluable support. Kevin Shannon, Dorothy Paderanga, Patricia Thompson, as well as collaborators Melvin Freedman, Karen Stephens, Eric Sievers, Maria Pallavicini, Molly Weaver, and Tom Grunberger. This work was funded by summer student research fellowships from the UCSF School of Medicine and UCSF Pediatric Clinical Research Center. A paper describing the major findings of this work has been published (Miles et al. *Blood* 88:4314, 1996)

Introduction

Neurofibromatosis type I (NF1) is a common autosomal dominant disorder with an incidence of 1 in 3000-4000.[1,3] NF1 (also known as von Recklinghausen's disease) primarily affects cells derived from the embryonic neural crest.[1] Patients with NF1 are predisposed to various benign and malignant tumors of the central nervous system (CNS), peripheral nervous system (PNS) and other tissues.[1-5] Children (but not adults) with NF1 are at markedly increased risk for developing malignant myeloid disorders, including juvenile chronic myelogenous leukemia (JCML) (recently renamed juvenile myelomonocytic leukemia (JMML)), bone marrow monosomy 7 syndrome (Mo 7) and acute myelogenous leukemia (AML).[3-6] The NF1 gene (*NF1*) was cloned in 1990[7-9] and encodes a 327 kDa protein called neurofibromin. The *NF1* gene contains 59 exons and is located on chromosome 17 at band q11.2[10]

Discovery of the *NF1* gene made it possible to examine the tumors from patients with NF1 for germline and somatic alterations. However, the diagnosis of NF1 remains a clinical one. NF1 is diagnosed when an individual exhibits two or more of the following symptoms: (1) six or more cafe-au-lait macules more than 5 mm in greatest diameter in prepubertal persons and more than 15 mm in greatest diameter in postpubertal persons, (2) two or more neurofibromas, (3) freckling in the axillary or inguinal regions, (4) optic glioma, (5) two or more Lisch nodules (iris hamartomas), (6)

a distinctive osseous lesion, and (7) a first degree relative with NF1 by the above criteria.[3] Phenotypically, NF1 has several unique characteristics. The disorder exhibits *age dependent expression;* multiple cafe-au-lait spots develop in childhood but cutaneous neurofibromas generally do not appear until puberty and adulthood. Moreover, the predisposition to malignant myeloid disorders seen in children is not observed in adults. Neurofibromatosis also exhibits *multiorgan expression* whereby patients often will show manifestations in the skin (freckling, cafe-au-lait macules), nervous system (tumors, retardation, learning disabilities, seizures, macrocephaly), skeletal system (kyphoscoliosis, short stature, tumors), and eye (Lisch nodules, congenital cataract). Lastly, NF1 has tremendous variability in the *severity of symptoms*. More than half the patients have relatively few symptoms and lead a normal life span, whereas others develop malignant tumors and/or have major physical or mental sequele.[2,3]

There have been few studies of long term survival in patients with NF1. One study in Denmark examined the natural history and long term survival of individuals with neurofibromatosis in a cohort of probands found through hospital records.[2] To reduce the possible bias in selecting a cohort of more severe NF1 patients who had been hospitalized, a cohort of their affected relatives (who were incidentally diagnosed) was also followed. Their study showed poorer survival rates for both men and women probands as well as their relatives as compared to the general population, although the survival curve for relatives was closer to that of the general population. This

epidemiologic study also highlighted the relationship between NF1 and tumorigenesis. In the control group, over half the tumors were found in three sites: the lung, prostate, and colorectum in men, and in the breast, colorectum and uterus in women. However, in the NF1 cohort, central nervous system tumors ranked first and nervous system tumors accounted for 47 percent of all malignant conditions. Gliomas accounted for more than 84 percent of the 19 documented CNS tumors and, interestingly, two osteosarcomas were described in the maxillary bone which is considered to arise from the neural crest. While the study showed a significant decrease in survival for the probands, relatives who were diagnosed incidentally showed only modest reduction in survival compared to the general population.[2] These findings emphasize the clinical variability and good prognosis of NF1. Although the relative risk for certain unusual tumors is greatly increased in patients with NF1, these fortunately are infrequent complications. However, the relationship between NF1 and cancer remains important.

A study by Matsui et al.[3] of 26,084 children with cancer found the frequency of children with NF1 was increased seven fold over what would be expected given the frequency of NF1 in the general population. The proportion of patients with NF1 was extremely high in optic glioma (12.5%) of all cases, malignant schwannoma (31.4%), rhabdomyosarcoma (1.36%) and myelogenous leukemia (0.27%) as compared to the expected rates.[3] Children with NF1 were also predisposed to pheochromocytomas, fibrosarcomas, and neurofibromas.[1-4] From these studies it was postulated that NF1 might

function to regulate growth control in cells derived from the embryonic neural crest. However, children with NF1 are also at increased risk for developing myelogenous leukemia, a cell which arises from mesenchymal origin. In a study by Stiller et al.[5] the relative risk in children with NF1 for myelogenous leukemia was 221 (95% CI 71-514). Children with NF1 were also predisposed to monosomy 7 syndrome (Mo 7) and acute myelogenous leukemia (AML).[6] A series of discoveries made about the biochemical activity of neurofibromin has lead to clues on how *NF1* might regulate growth in hematopoietic and neural crest cells.

In order to divide, a cell must enter and navigate through several critical progression points where an multitude of competing factors and signals interact to either inhibit or drive cells through the cell cycle. A resting cell in G_0 state must be signaled or stimulated to enter into G_1 then proceed through DNA synthesis (S phase), G_2 and finally mitosis or M phase. Changes in the activity of regulatory factors can lead to apoptosis where a cell is programmed to die, or unrestrained malignant growth. $p21^{ras}$ (Ras), a member of a large family of small guanine nucleotide triphosphate (GTP) binding proteins, is believed to play a crucial role in signaling pathways that are involved in growth control.

Ras belongs to a family of GTP binding proteins that serve a wide variety of regulatory function in mammalian cells. These proteins regulate cell growth by binding and hydrolyzing GTP. Mammalian *RAS* genes encode three 21 kDa protein products that consist of a N-terminus catalytic domain,

that is required for nucleotide exchange and effector function, a C-terminus for attachment to the plasma membrane, and an third linking segment. The three mammalian Ras proteins (H-Ras, K-Ras, and N-ras) all have slow and weak intrinsic biochemical activity both to bind GTP and to hydrolyze the phosphate bond and convert GTP to guanine nucleotide diphosphate (GDP). It is through the hydrolysis of this bond that Ras proteins regulate their activity; Ras-GTP is the active form and Ras-GDP is inactive. The ability of Ras proteins to cycle efficiently between active and inactive conformations comes from their interactions with guanine nucleotide exchange proteins (which stimulate the conversion of Ras-GTP to Ras-GDP) and GTPase activating proteins (GAPs) which negatively regulate Ras by stimulating the hydrolysis of GTP to GDP, see Figure 1.

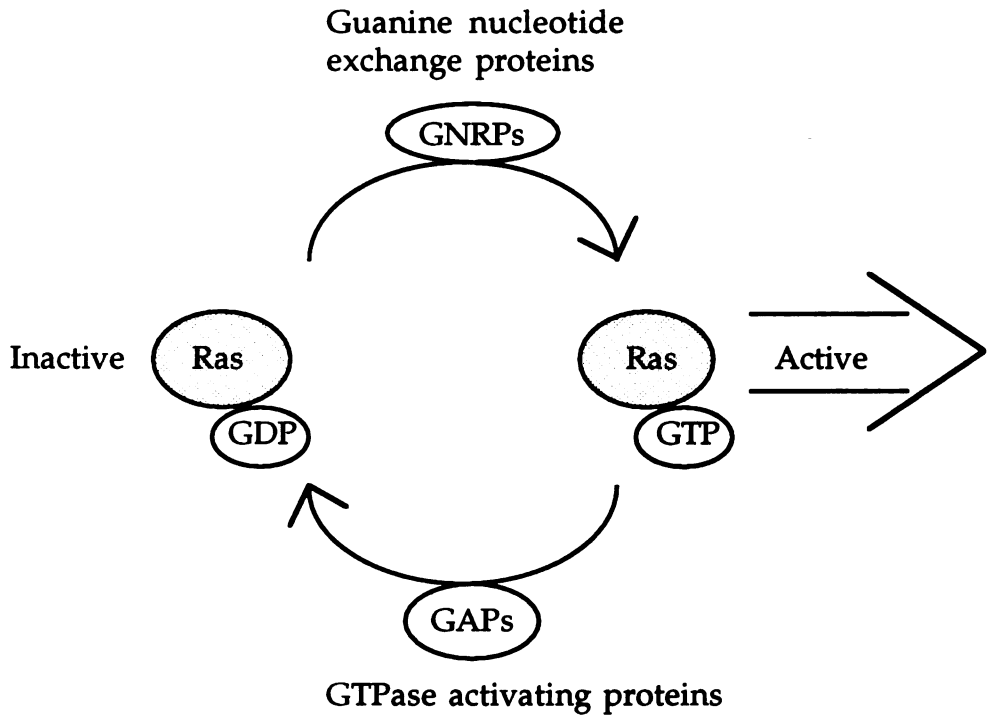

Figure 1. Ras cycle. Ras becomes active when bound to GTP and becomes inactive when bound to GDP. GAPs down regulate Ras by binding it and facilitating hydrolysis of GTP to GDP.

A large body of evidence implicates Ras proteins in normal and abnormal hematopoietic cell growth.[11,12] Normally, only a small proportion of Ras is bound to GTP; in contrast oncogenic Ras proteins accumulate in the GTP-bound conformation. Activating *RAS* mutations are common in myeloid leukemia, including the bone marrows of children with JMML, Mo 7 or AML.[13-18] These mutations impair the intrinsic GTPase activity of Ras proteins and block the ability of GAPs to stimulate GTPase activity, thereby elevating the percentage of Ras in the active GTP-bound state.[12] This constitutively active state causes cells (through mechanisms that are not

clearly understood) to lose growth control and become neoplastic. Moreover, mutant RAS alleles efficiently transform myeloid cell lines.[19,20] Positional cloning of the *NF1* gene has revealed an unexpected relationship between *NF1* and Ras.

A segment of the *NF1* gene was found to share sequence homology with a highly conserved catalytic domain within mammalian p120GAP (rasGAP), as well as to other GTPase activating proteins, including yeast IRA1 and IRA2, Drosophila GAP1 and Saccharomyces pombe gap1. Furthermore, expression of this conserved GAP-related domain (GRD) of neurofibromin revealed that it can down regulate Ras by stimulating it's intrinsic GTPase activity.[21] These data suggested that neurofibromin restrains cell growth by down-regulating Ras. Analysis of *NF1* like genes in lower organisms supported this hypothesis. Deletions of IRA1 and IRA2 or gap1 resulted in increased levels of GTP-bound Ras and produced phenotypes similar to strains containing activated RAS mutations.[22] Li et al.[23] detected amino acid substitutions altering lysine 1423 of neurofibromin not only in an NF1 family, but three sporadic tumors: an astrocytoma, a colon carcinoma and in a myelodysplastic bone marrow. Lysine 1423 is one of the 14 amino acids in the neurofibromin catalytic domain which is absolutely conserved across all members of the GAP family. The GAP activity of the mutant GRD was found to be 200 to 400 times lower than that of the wild type, while binding affinity was unaltered. Furthermore, recent biochemical and cell culture studies of leukemic bone marrows cells from children with NF1, and of hematopoietic

cells harvested from embryos with homozygous inactivation of the murine NF1 gene (*Nf1*) have shown elevated levels of Ras-GTP, a decrease in the ability to accelerate GTP hydrolysis on Ras, and activation of mitogen activated protein (MAP) kinase (a downstream effector of Ras). [24,25]

Is neurofibromin solely a GTPase-activating protein, or might neurofibromin exhibit addition mechanisms for growth control? Many have speculated that neurofibromin may perform a variety of functions in different cell types. Interestingly, some neural crest cell tumors that lacked neurofibromin were found to have normal levels of Ras-GTP[26], and while aberrations in the *NF1* gene has been found in both neuroblastoma and melanoma cell lines[27], patients with NF1 are not predisposed to these cancers Recent work on p120GAP may provide addition evidence for alternative functions of neurofibromin. Studies showed an association of p120GAP with a tyrosine kinase receptor and two phosphotyrosine containing proteins p62 and p190 upon stimulation of cells with growth factors[28,29]; p62 shows extensive sequence homology to an heteronuclear ribosomal nuclear protein (hnRNP) GRP33, and sequence analysis of p190 showed that it may itself function as a GTPase and GTP binding protein. It was also discovered that the central region of p190 encoded a transcriptional repressor of the glucocorticoid receptor gene.[28.29] Taken together, these findings, as well as the multi-organ involvement of NF1 and large structure of neurofibromin, implicate possible Ras-independent functions of neurofibromin.

The biochemical features of neurofibromin and the dominantly-inherited cancer predisposition seen in patients with NF1 suggest that *NF1* might function as a tumor suppressor gene. The theory behind tumor suppressor genes grew out of the observation that non-oncogenic cell lines fused to oncogenic ones could transfer genetic material capable of suppressing the neoplastic phenotype of their tumor cell partners. From this, the discovery that proteins existed that down regulate growth was made. The retinoblastoma (*RB1*) gene was the first tumor suppressor gene discovered; it has been implicated in tumors of the retina, lung, soft tissue and bone.[30] Through epidemiologic studies of retinoblastoma, Knudson developed the first model behind the existence of tumor suppressor genes known as the Knudson model (Figure 2). While the predisposition to cancer is inherited as a dominant trait, tumor suppressor alleles act in a recessive fashion at the cellular level with loss of both alleles required for deregulated growth control. This may partly explain the poor penetrance and clinical variability observed in NF1. For example, although a group of individuals may all have NF1 and inherit one inactivated *NF1* allele, a second somatic mutation may never occur and thus an individual may never develop cancer. Knudson postulated that two forms of disease existed, sporadic and familial. Patients with the familial pattern are predisposed to developing tumors because they carry a germline mutation in one allele in every cell. However, in the sporadic form two independent somatic mutations must occur within the same cell, an event that would be expected to occur with much less frequency.

Accordingly, the majority of familial retinoblastoma was associated with bilateral eye tumors whereas sporadic cases were always unilateral.

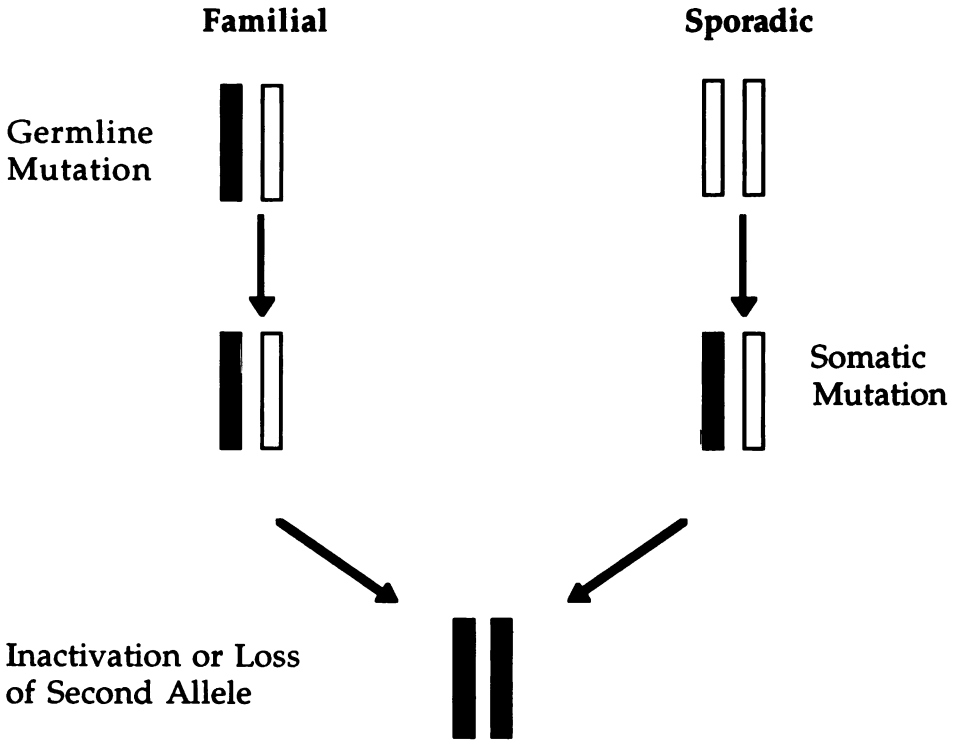

Figure 2. The Knudson model. In familial cases, individuals are predisposed to cancer because one germline mutation is inherited in every cell and all that is required for loss of function is a single somatic event. In sporadic cases, two independent somatic events or "hits" must occur within the same cell for deregulated growth.

Tumor suppressor genes have been found to play major roles in cell cycle control, and a number of other suspected tumor suppressor genes have been cloned in various cancers in addition to *RB1* and *NF1*.[31] Molecular analysis of tumors from patients with NF1 support it's role as a tumor suppressor gene. Xu et al.[32] reported loss of one *NF1* allele in each of 7 pheochromocytoma tumors from NF1 patients. In each of the three cases for which it could be determined, the wild type *NF1* allele was lost. In addition, Skuse et al.[33] described loss of heterozygosity (LOH) at the *NF1* locus in malignant peripheral nerve tumors of NF1 patients. Furthermore, a limited study by Shannon et al.[34] described loss of the normal *NF1* allele in the leukemic marrows of 4 children with NF1 and myeloid leukemia.

Taken together, these results support the hypothesis that *NF1* functions as tumor suppressor gene in certain neural crest cells and immature myeloid cells. Although the normal *NF1* allele was absent in leukemic bone marrows of a few children with NF1, there are no data that address which hematopoietic lineages were involved in these malignant clones. In this study we utilized Epstein-Barr virus (EBV) transformed cell lines, erythroblasts plucked from burst forming unit-erythroid (BFU-E) colonies, and $CD34^+$ stem cell populations from leukemic marrows with allelic losses at *NF1* to provide evidence that malignant transformation in JMML occurs in an immature myeloid progenitor that is committed to myeloid differentiation. Our data also extend the previous observation that

boys who inherit NF1 from their mothers are at the highest risk of developing myeloid disorders.[34]

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. Children were diagnosed with JMML by pediatric hematologists at the referring institutions using a combination of clinical and laboratory criteria[35] including: (1) leukocytosis with an elevated absolute monocyte count; (2) presence of immature myeloid precursors in the peripheral blood; (3) <30% blasts in bone marrow aspirates; and (4) 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 (CFU-GM) to granulocyte-macrophage colony stimulating factor (GM-CSF).

Polymorphic Markers in the NF1 Region

Investigating these leukemic samples for allelic loss was complicated by the fact that normal non-hematopoietic tissue from the same patients were

not available to determine if their bone marrows showed loss of constitutional heterozygosity 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. Peripheral blood cells were lysed by mixture of equal volumes of blood with (0.14M NaCl) and 5 drops of zap-oglobin II lytic reagent (Coulter). Cells were then placed on ice for 5 minutes and centrifuged at 2500 to 3500 rpm for 15 minutes and washed twice with (0.14M NaCl). Samples were resuspended in STE buffer, proteinase K (final concentration 100ug/mL) and SDS (final concentration 0.5%); samples were then shaken overnight at 55^{0}C. An equal volume of phenol/chloroform solution was added for 10 minutes; samples were then centrifuged at 2500 rpm and to the aqueous layer 1 mL of 3M NaOAc per 10 mL of sample was added and two volumes of 100% EtOH. Samples were stored at -20^{0}C for six hours centrifuged and washed twice with 70% EtOH. Pellets were resuspended in 3 mL TE Buffer and treated with RNase to a final concentration of 100ug/mL at 37^{0}C for 45 minutes. Proteinase K and SDS were added to samples as above and incubated at 55^{0}C for 1 hour. Samples then underwent phenol/chloroform extraction as described previously. Bone marrow samples were suspended in an equal volume of RPMI 1640 media and 10% fetal bovine serum (FBS) and layered onto 3 mL of ficoll (Histopaque-1077) solution. Tubes were spun for 20 minutes at 2000 rpm, buffy coat was removed and cells were suspended in 15 mL RPMI 1640 and 10% FBS, and centrifuged at 100 rpm for 7 minutes; cells were

resuspended in 1 mL of media and 10% FBS. DNA was then extracted as described for peripheral blood.

We routinely utilized 3 sequence polymorphisms within *NF1* and 1 marker located centromeric of the gene to screen for allelic loss. The intragenic markers are EVI-20[34], an Alu repeat described by Xu et al.[36] and a complex repeat described by Andersen et al.[37]; the flanking marker is UT-172.[34] 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.[38] 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 Cetus). PCR was performed in reaction mixtures that include 10 pM of respective 3' and 5' primers, 100 ng of target genomic DNA, 1 unit of Taq polymerase (AmpliTaq, Cetus Corp.), 2mM magnesium chloride and 100 mM final concentrations of deoxynucleotides in a final reaction volume of 25 µL. ^{32}P deoxy-ATP was incorporated into the DNA fragments generated in the PCR procedure by adding 1 µL (10 µCi) of ^{32}P deoxy-ATP per 25 µL of the reaction mixtures and decreased the concentrations of "cold" deoxy-ATP to 50 mM. Labeled PCR products were separated on (6M urea, 8 percent polyacylamide) sequencing-type gels and run at 60-80 watts constant power for 2-4 hours. The gels were placed in Saran wrap and exposed to X-ray film for 1-

36 hours at -70°C. Amplification of EVI-20 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. EVI-20 detects four common alleles ranging from 200-210 base pairs (bp). PCR conditions for Alu consisted of 30 cycles of denaturation at 94°C for one minute, annealing at 52°C for one minute, and extension at 72°C for one minute. Alu detected five alleles ranging from 403-390 bp. UT-172 conditions were 35 cycles of denaturation at 94°C for 45 seconds, annealing at 52°C for 45 seconds, and extension at 72°C for 45 seconds. UT-172 detected four alleles ranging from 100-120 bp. Amplification of UM (data not shown) was performed as described in Andersen et al.[37], amplification of IVS27AG and IVS27TG (data not shown) were as described in Lazaro et al.[38]; UM amplified five alleles ranging from 266-235 bp; IVS27TG amplified five common alleles from 270-292 base pairs and IVS27AC amplified seven alleles from 207-219 bp.

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), resuspended in 1 mL of RPMI 1640 medium supplemented with 20% fetal bovine serum, L-

glutamine and antibiotics, and the number of viable lymphocytes was 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.) that had been prepared by washing 4 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 non adherent cells were then recovered by gently rocking the flask and removing the cell suspension. The cells were washed and re-suspended at 1 x 10^6 cells per mL. A total of 0.1 mL of EBV supernatant from cell line B95-8 cell line was added per 1 x 106 cells with thorough mixing. Cells were cultured at 1-2 x 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 mM Hepes, L-glutamine, and antibiotics. Transformation was ascertained by the appearance of tightly formed cell clusters and usually occurred within 3-6 weeks. The EBV cell line from the patient shown in Figure 4 was prepared using a modification of the method of Neitzel.[39]

Isolation of CD34+ Cells

Frozen cells were thawed gradually and washed once in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal calf serum prewarmed to 37^{0}C. Nucleated cells were labeled with 7.5 μM Hoechst

33342 (HO) at 37^{0}C for 60 minutes, washed in Hank's balanced salt solution containing 0.02% azide, and incubated at 4^{0}C for 45 minutes with fluorescein isothiocyanate-conjugated (FITC) anti-CD34 antibody (Becton Dickinson (BD), San Jose CA). Following antibody labeling, the cells were washed once with phosphate buffered saline. Dead cells were stained with 1 μg/mL propidium iodide (PI; Calbiochem, Santa Clara CA) for 5 minutes at 20^{0}C. Flow cytometric analysis was performed using a FACStar PLUS (BD) equipped with 2 argon lasers tuned to 488 nm and 351-364 nm (ultraviolet 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 emission (ultraviolet excitation) were collected through a 425 nm (+ 50 nm) bandpass and a 620 nm long pass filter, respectively. PIdim/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 immunofluoresence intensity above which cells were considered labeled. PIdim/HO-positive CD34$^+$ cells were sorted into a test tube containing RPMI medium and DNA was extracted as described previously. The small numbers of bone marrow mononuclear cells available from these young children precluded utilizing 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 myeloproliferative syndrome (MPS) that did not satisfy diagnostic criteria for JMML. DNA was available from both of the parents for patients 2, 3, 5, 7, 8, 9, and 10; from one parent for patients 1, 4, and 6; and from neither parent for patient 11. We demonstrated allelic loss at *NF1* in the bone marrows of patients 1, 3, 7, and 8. Three of these children had familial NF1 and in each case the allele inherited from the unaffected parent was deleted. Representative data are shown in Figure 3. The JMML bone marrow of patient 6 showed a single fragment with all of the polymorphic markers tested and consistently retained an allele that he shared with his mother and maternal grandmother, both of whom have NF1 (data not shown). All 3 affected members of this family shared an uncommon 235 bp fragment amplified with the primers described by Andersen et al.[38] and this allele was not present in an unaffected sibling. These data suggest that the normal NF1 allele was deleted from this child's marrow. Similarly, the bone marrow of patient 11 showed a single fragment with all 6 polymorphic markers and it is therefore likely that the bone marrow has deleted an *NF1* allele. The probability that an individual will be uninformative at all of these loci is

< 2% and every parental DNA sample studied to date has exhibited two alleles with at least one of the markers.

TABLE 1

Clinical Features of Study Population

Patient No. and Sex	Age at Onset	Diagnosis	Parent with NF1	Results with NF1 Markers
1M	24 mons	Monosomy 7	Unknown	LOH
2M	6 mons	JMML	Neither	HZ
3M	14 mons	JMML	Mother	LOH
4M	6 mons	JMML	Mother	HZ
5M	22 mons	AMMoL	Neither	HZ
6M	30 mons	JMML	Mother	probable LOH
7M	60 mons	JMML	Mother	LOH
8F	30 mons	JMML	Mother	LOH
9M	19 mons	MPS	Mother	HZ
10F	12 mons	JMML	Neither	HZ
11F	18 mons	JMML	Unknown	probable LOH

Abbreviations Used: M - male; F - female; LOH - loss of heterozygosity; HZ - retains heterozygosity; mons - months; Mo 7 - monosomy 7 syndrome; JMML - juvenile myelomyelomonocytic leukemia; AMMoL - acute myelomonocytic leukemia.

We developed EBV cell lines from 5 patients including 3 with allelic losses at *NF1* (patients 3, 7, and 8); isolated $CD34^+$ cells from the frozen bone marrow mononuclear cells of patients 3, 7, and 8; and plucked erythroblasts from immature erythroid (BFU-E) colonies grown from the bone marrow of patient 3.

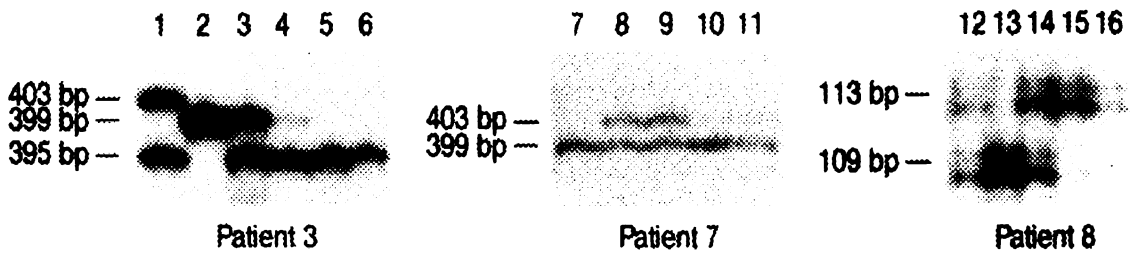

Figure 3. Analysis of bone marrow and EBV cell line DNA from patients 3, 7, and 8 and DNA extracted from the blood of their parents. The mother is affected with NF1 in all 3 families. Lanes 1-11 were analyzed with the marker described by Xu[41] and lanes 12-16 were analyzed with UT172. Lanes 1, 7 and 12 show maternal DNA, lanes 2, 8 and 13 show paternal DNA, lanes 3, 9 and 14 show EBV line DNA and 4, 10 and 15 show DNA extracted from leukemic bone marrow. Lanes 5, 11 and 16 represent DNA isolated from $CD34^+$ cells in patient marrow and lane 6 shows analysis of BFU-E from one affected child. In all 3 cases, polymorphic *NF1* markers demonstrate that the EBV lines have retained both parental alleles whereas the affected bone marrow samples have deleted the normal paternal *NF1* allele.

As shown in Figure 3, the EBV cell lines of these 3 patients retained both *NF1* alleles while the $CD34^+$ cells consistently demonstrated loss of constitutional heterozygosity. DNA extracted from the BFU-E colonies of patient 3 also showed LOH. As patients 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 demonstrate involvement of erythroid progenitors in the abnormal clone. An EBV line was also available from a child with a myeloproliferative syndrome (MPS) reported previously (patient 8).[34] 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 (Figure 4). 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 per mm^3, 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. Cytogenetic 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.

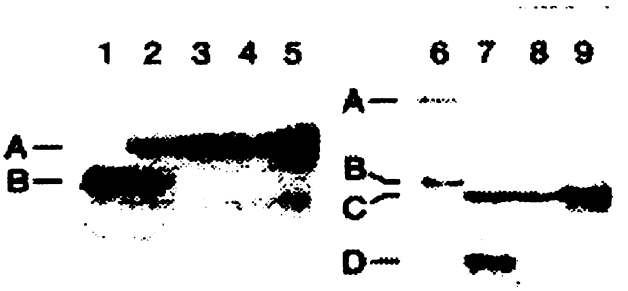

Figure 4. Analysis of bone marrow and EBV cell line DNA from a child who developed an unusual MPS with the polymorphic markers UT172 (lanes 1-5) and D17S787 (lanes 6-9). Different alleles are designated in order of size beginning with the letter "A". DNA from the following sources were amplified and resolved: Lanes 1 and 6: mother; Lanes 2 and 7: father; Lanes 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.[38]

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). By chi square analysis, there was a significantly greater proportion of mothers transmitting NF1 to children who developed leukemia ($p<0.02$) and a significantly greater proportion of affected boys with familial NF1 ($p < 0.01$).

TABLE 2

Parental Transmission of Familial NF1 in Children with Myeloid Disorders*

	Sex of Parent with NF1	
Sex of Affected Child	**Mother**	**Father**
Male	19	8
Female	4	1

* - Includes the patients in this study; cases described previously as reviewed in Shannon et al.[6] and cases in Shannon et al.[35]

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 germ line

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.[30,31] *NF1* fulfills all of these criteria in myeloid leukemia. First, children with NF1 are at markedly increased risk of developing malignant myeloid disorders, particularly JMML.[4-6,34] Second, when data from the patients included in this study are combined with cases in a previous report[34], 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.[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 NF1[20], and direct evidence that the Ras signaling pathway is activated in leukemic cells from children with NF1 and in murine *Nf1-/-* hematopoietic cells[24] provide strong evidence that neurofibromin normally restrains the growth of myeloid cells by negatively regulating Ras.

Formal proof that *NF1* functions as a tumor suppressor gene depends on loss of both *NF1* alleles within tumor cells. Dr. Lucy Side, a post-doctoral fellow in our lab recently found truncating mutations in the *NF1* gene in children with NF1 and myeloid leukemias whose tumors showed loss of the normal *NF1* allele.[40] Furthermore, the mutant proteins were not expressed *in vivo*. These data prove that both *NF1* alleles are inactivated within myeloid leukemia. This, along with the evidence of loss of the normal *NF1* allele in both pheochromocytomas and peripheral nerve tumors in NF1 patients[32,33] confirm the role of *NF1* as a tumor suppressor gene in myeloid and certain neural crest cell types. Additionally, mice reconstituted with *Nf1-/-* cells develop a myeloproliferative disease and exhibit a prolonged rise in Ras-GTP after stimulation with granulocyte/macrophage colony stimulating factor (GM-CSF).[25] This agrees with clinical observations that leukemic cells from children with JMML will exhibit hypersensitivity to GM-CSF *in vitro*.[41]

However, several lines of evidence suggest that *NF1* may function in growth control through mechanisms that are independent of it's role as a GAP for Ras, particularly in neural crest cell lineages. First, whereas Ras mutations are found in the marrows of 20-40% of children with myelogenous leukemia[13-16], there is a low incidence of *RAS* mutations found in sporadic neural crest cell tumors.[20] One might expect to find a higher number of RAS mutations in neural crest cell tumors if elevated Ras-GTP plays a central role in tumorgenesis in those cell types. Secondly, although neural crest and sensory neurons isolated from *Nf1-/-* embryos exhibited growth factor

independence and survived in the absence of nerve growth factors (where their wild-type counterparts died rapidly),[42] in PC12 cells and other neuronal cell types oncogenic Ras resulted in neuronal cell differentiation instead of proliferation.[43,44] Furthermore, in contrast to cell lines derived from malignant schwannomas from NF1 patients that lacked neurofibromin and exhibited abnormally elevated levels of Ras-GTP[45], neuroblastoma and melanoma cell lines that lacked neurofibromin surprisingly showed normal levels of Ras-GTP.[26] In fact, representative cell lines from these tumors that over expressed Ras still contained sufficient GTPase activity to negatively regulate Ras. It is also interesting that while neuroblastoma and melanoma cells are both derivatives of the neural crest, patients with NF1 are not predisposed to these tumors.[1-5] Perhaps in cell types that may be able to regulate Ras through other GAPs such as p120GAP other modifying factors are needed in addition to inactivation of *NF1* for tumorigenesis. In contrast, malignancies where regulation of Ras is predominantly by *NF1*, loss of *NF1* alone may be sufficient to cause malignant transformation. Taken together, these data suggest an alternative growth regulatory function in addition to GAP activity for neurofibromin in some types of cells.

JMML is the most common leukemia that arises in children with NF1.[34] The clonal nature of this disorder is supported by studies of rare patients with a marker cytogenetic alteration in the bone marrow[46] and, more recently, by demonstrating a clonal pattern of hematopoiesis by the criteria of a non-random pattern of X chromosome inactivation in girls with JMML.[47]

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 where involvement of both B lymphocytes and myeloid cells has been shown by a number of approaches.[48-51] The myelodysplasias associated with monosomy 7 or del(5q) are similar to JMML in that the cytogenetic abnormality is apparently restricted to myeloid cells.[52,53] 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.[54,55] In addition, JMML bone marrows contain a higher percentage of $CD34^+$ cells than normal samples (mean 4.9%; range 3.8 - 5.8% in JMML versus mean 1.8%; range 0.6 - 2.9% in normal marrows; M.H. Freedman and T. Grunberger; unpublished data). These findings are consistent with data from mice reconstituted with *Nf1*-deficient hematopoietic cells which develop a JMML-like syndrome that is associated with a marked increase in the percentage of

immature Mac-1^{+}, Gr-1^{lo} myeloid progenitor cells in the marrow.[25] While all of these data demonstrate a myeloid/erythroid pattern of involvement in JMML, it is possible that this disorder arises from 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*.[25] 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.[46] Papayannopoulou and associates[56] 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.[57] 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.[56,57] Our data showing loss of the normal *NF1* allele in erythroblasts harvested from the BFU-E colonies of a JMML patient confirm this hypothesis.

While EBV-transformed cells from patients with JMML consistently retained both *NF1* alleles, we observed allelic loss in both unfractionated bone marrow and EBV-transformed cells from a child with an atypical MPS. This case demonstrates that loss of *NF1* 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 as CML is associated with activation of the Ras signaling pathway through binding of Bcr-Abl to the Grb2 adapter protein.[58] Transfection of *BCR-ABL* into hematopoietic and non-hematopoietic cell lines results in phosphorylation of other proteins in the Ras signaling pathway including p120GAP, MAP kinase, and Jun N-terminal kinase[59-61] and recent data implicate Ras activation as essential for *BCR-ABL* induced transformation.[61] Additional experiments are required to characterize the full spectrum of hematopoietic lineage involvement in the myeloid disorders associated with NF1 and to ascertain if the level at which transformation occurs correlates with clinical behavior.

Leukemic transformation is much less common in children with NF1 than in patients who inherit germ line mutations that predispose to retinoblastoma or Wilms' tumor[34,35] This suggests that loss of the normal *NF1* allele occurs very infrequently in hematopoietic cells or, alternatively, that inactivation of *NF1* 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 *Nf1-/-* fetal liver cells consistently develop a JMML-like disorder[25] suggests that inactivation of *Nf1* is sufficient to deregulate myeloid growth. However, other evidence support the idea that loss of *NF1* function interacts with other genetic factors in human leukemogenesis. First, some NF1 patient bone marrows show cytogenetic

abnormalities, particularly monosomy 7.[6,61] Second, epidemiologic data implicate epigenetic factors in the development of myeloid disorders in children with NF1. According to the Knudson model, inheriting a germ line *NF1* mutation should predispose to cancer in a manner that is independent of the sex of either the transmitting parent or the affected child.[30,31] This is not true in children with NF1 as boys who inherit NF1 from their mothers are at especially high risk of developing myeloid disorders. While the male predilection seen among children with NF1 is similar to the distribution of cases of JMML and Mo 7 in the general population[34,63,64], 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 NF1 from their mothers is unexpected and is not easily explained. The proposal that the maternal NF1 allele might be imprinted in hematopoietic cells (reviewed in[65,66] is not supported by our finding that mutant *NF1* alleles of both maternal (6 cases) and paternal (3 cases) origin have been retained in patient bone marrows (23, and these data).

The results of this study firmly establish *NF1* as a tumor-suppressor gene in immature hematopoietic cells and raise a number of questions with respect to the role of *NF1* inactivation in leukemogenesis. The reason children with NF1 are strongly predisposed to myeloid disorders while 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 *Nf1* gene have only been observed in adult animals[64] In addition, while 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 NF1 and the nature of the genetic events that cooperate in leukemogenesis requires further study.

References

1. Riccardi VM, Eichner JE: Neurofibromatosis. Baltimore, MD, Johns Hopkins, 1986

2. Sorensen SA, Muvihill JJ, Nielsen A: Long-term follow up of von Recklinghausen Neurofibromatosis. *The New England Journal of Medicine* 314:1010, 1986

3. Matsui I, Tanimura M, Kobayasi N, Sawada T, Nagahara N and Akatsuka J: Neurofibromatosis type 1 and childhood cancer. *Cancer* 72:2746, 1993

4. Bader JL, Miller RW: Neurofibromatosis and childhood leukemia. *Journal of Pediatrics* 92:925, 1978

5. Stiller CA, Chessells JM, Fitchett M: Neurofibromatosis and childhood leukemia/lymphoma: a population-based UKCCSG study. *British Journal of Cancer* 70:969, 1994

6. Shannon KM, Watterson J, Johnson P, O'Connell P, Lange B, Shan N, Kan YW, Priest JR: Monosomy 7 myeloproliferative disease in children with neurofibromatosis, type 1: Epidemiology and molecular analysis. *Blood* 79:1311, 1992

7. Cawthon RM, Weiss R, Xu GF, Viskochil D, Culver M, Stevens J, Robertson M, Dunn D, Gesteland R, O'Connell P, White R: A major segment of the neurofibromatosis type 1 gene: cDNA sequence, genomic structure and point mutations. *Cell* 62:193, 1990

8. Viskochil D, Buchberg AM, XU G, Cawthon RM, Stevenrs J, Wolff RK, Culver M, Carey JC, Copeland NG, Jenkins NA, White R, O'Connell P: Deletions and a translocation interrupt a cloned gene at the neurofibromatosis type 1 locus. *Cell* 62:187, 1990

9. Wallace MR, Marchuck DA, Anderson LB, Letcher R, Odeh HM, Saulino AM, Fountain JW, Brereton A, Nicholson J, Mitchell Al, Brownstein BH, Collins FS: Type 1 neurofibromatosis gene: Identification of a large transcript disrupted in three NF1 patients. *Science* 249:181, 1990

10. Baker D wright E, Nguyen K, cannon L, Fain P, Goldgar D, Bishop DT, Carey J, Baty B, Kivlin J, Willard H, Waye JS, Greig G, Leinwand L, Nakamura Y, O'Connell P, Leppert M, Lalouel J-M, White R, Skolnick M: Gene for von Recklinghausen neurofibromatosis is in the pericentromeric region of chromosome 17. *Science* 236:1100, 1987

11. Boguski M, McCormick F: Proteins regulating ras and it's relatives. *Nature* 366:643, 1993

12. Hall A: The cellular functions of small GTP-binding proteins. *Science* 249:635, 1990

13. Lubbert M, Mirro J, Kitchingman G, McCormick F, Mertelesmann R, Herrmann F, Koeffler P: Prevelance of N-ras mutations in children with myelodysplastic syndrome and acute myeloid leukemia. *Oncogene* 7:263, 1992

14. Neubauer A, Shannon KM, Lui E: Mutations of the ras protooncogenes in childhood monosomy 7. *Blood* 77:594, 1991

15. Miyauchi J, Asada M, Sasaki M, Tsunematsu Y, Kojima S, Mitzutai S: Mutations of the *N-ras* gene in juvenile chronic myelogenous leukemia. *Blood* 83:2248, 1994

16. Kalra R, Paderanga D, Olson K, Shannon KM: Genetic analysis consistant with the hypothesis that NF1 limits myeloid cell growth through $p21^{ras}$. *Blood* 84:3435, 1994

17. Bos Jl: *ras* oncogenes in human cancer: A review. *Cancer Research* 49:4682, 1989

18. Rodenhuis S: ras and human tumors. *Seminar in Cancer Biology* 3:241, 1992

19. Pierce JH, Aaronson SA: Myeloid cell transformation by *ras*-containing murine sarcoma viruses. *Molecular and Cell Biology* 5:667, 1985

20. Kahn P, Frykberg L, Brady C, Stanely I, Beug H, Vennstrom B, Graf T: v-erbA cooperates with sarcoma oncogenes in leukemic cell transformation. *Cell* 45:349, 1986

21. Hall A: Signal transduction through small GTPases-a tale of two GAPs. *Cell* 69:389, 1992

22. Tannaka K, Nakafuku M, Satoh T, Marshall MS, Gibbs JB, Matsumoto K, Kaziro Y, and Toh-e A. *Cell* 60:803, 1990

23. Li Y, Bollag G, Clark R; Stevens J, Conroy L, Fults D, Ward K, Friedman E, Samowitz W, Robertson M: Somatic mutations in the neurofibromatosis 1 gene in human tumors. *Cell* 69:275, 1992

24. Bollag G, Clapp DW, Shih S, Adler F, Zhang Y, Thompson P, Lange BJ, Freedman MH, McCormick F, Jacks T, Shannon K: Loss of *NF1* results in activation of the Ras signaling pathway and leads to aberrant growth in murine and human hematopoietic cells. *Nature Genetics* 112:144, 1996

25. Largaespalda DA, Brannan CI, Jenkins NA, Copeland NG: Nf1 deficiency causes Ras-mediated granulacyte-macrophage colony-stimuating factor hypersensitivity and chronic myeloid leukemia. *Nature Genetics* 12:137, 1996

26. Johnson MR, Look AT, DeClue JE, Valentine MB, Lowry DR: Inactivation of the *NF1* gene in human melanoma and neuroblastoma cell lines without impaired regulation of GTP-Ras. *Proceedings of the National Academy of Science USA* 90:5539, 1993

27. The I; Murthy AE; Hannigan GE; Jacoby LB; Menon AG; Gusella JF; Bernards A: Neurofibromatosis type 1 gene mutations in neuroblastoma. *Nature Genetics* 3:62-6, 199332.

28. Wong G; Müller O; Clark R; Conroy L; Moran MF; Polakis P; McCormick F: Molecular cloning and nucleic acid binding properties of the GAP-associated tyrosine phosphoprotein p62. *Cell* 69:551, 1992

29. Settleman J, Narasimhan V, Forster LC, Weinberg RA: Molecular cloning of cDNAs encoding the GAP-associated protein p190: implications for a signaling pathway from ras to the nucleus. *Cell* 69: 1992

30. Weinberg RA: Tumor suppressor genes. *Science* 254:1138, 1991

31. Knudson AG: Antioncogenes and human cancer. *Proceedings of the National Academy of Science.* USA 90:10914, 1993

32. Xu W, Mulligan LM, Ponder MA, Liu L, Smith BA, Matthew CGP, Ponder BAJ: Loss of NF1 alleles in pheochromocytomas from patients with type 1 neurofibromatosis. *Genes, Chromosomes & Cancer* 4:337, 1992

33. Skuse GR, Kosciolek BA, Rowley PT: Molecular analysis of tumors in von Recklinghausen neurofibromatosis: Loss of heterozygosity for chromosome 17. *Genes, Chromosomes & Cancer* 1:36, 1989

34. Shannon KM, O'Connell P, Martin GA, Paderanga D, Olson K, Dinndorf P, McCormick F: Loss of the normal NF1 allele from the bone marrow of children with type 1 neurofibromatosis and malignant myeloid disorders. *New England Journal of Medicine* 330:597, 1994

35. Freedman MH, Estrov Z, Chan HSL, Juvenile Chronic Myelogenous Leukemia. *The American Journal of Pediatric Hematology/Oncology* 10:261, 1988

36. Xu G, Nelson L, O'Connell P, White R: An Alu polymorphism intragenic to the neurofibromatosis, type 1 gene. *Nucleic Acid Research* 19:3764, 1991

37. Andersen LB, Tarle SA, Marchuk DA, Legius E, Collins FS: A highly informative compound nucleotide repeat in the neurofibromatosis (NF1) gene. *Human Molecular Genetics* 2:1083, 1993

38. Lazaro C, Gaona A, Estivill X: Two CA/GT repeat polymorphisms in intron 27 of the human NF1 gene. *Human Genetics* 93:351, 1994

39. Neitzel H: A routine method for the establishment of permanent growing lymphoblastoid cell lines. *Human Genetics* 73:320, 1986

40. Side L, Taylor B, Cayouette M, et al.: Neurofibromatosis type 1 gene mutations in children with NF1 and malignant myeloid disorders (abstract). *Blood* 1996;88 (Suppl 1):477a.

41. Estrov Z, Grunberger T, Chan HSL, Freedman MH, Juvenile chronic myelogenous leukemia; characterization of the disease using cell cultures. *Blood* 67:1382, 1986

42. Vogel KS, Brannan CI, Jenkins NA, Copeland NG, Parada L: Loss of neurofibromin results in neurotropin-independent survival of embryonic sensory and sympathetic neurons. *Cell* 1995;82:733-742.

43. Noda M, Ko M, Ogura A: Sarcoma viruses carrying *ras* oncogenes induce differentiation-associated properties in a neuronal cell line. *Nature Genetics* 3:118, 1993

44. Bar-Sagi D, Feramisco JR: Microinjection of the *ras* oncogene protein into PC12 cells induces morphologic differentiation. *Nature* 9:73, 1985

45. DeClue JE, Papageorge AG, Fletcher JA, Diehl SR, Ratner N, Vass WC, Lowry DR: Abnormal regulation of mammalian p21ras contibutes to

malignant tumor growth in von Recklinghausen (type 1) neurofibromatosis. *Cell* 69:265, 1992

46. Amenomori T, Tomonaga M, Yoshida Y, Kuriyama K, Matsuo T, Jinnai I, Ichimaru M, Omiya A, Tsuji Y: Cytogenic evidence for partially committed myeloid progenitor cell origin of chronic myelomonocytic leukaemia and juvernile chronic myeloid leukaemia: both granulocyte-macrophage precursors and erythroid precursors carry identical marker chromosome. *British Journal of Haemotology* 64:539, 1986

47. Busque L, Gilliland DG, Prchal JT, Sieff CA, Weinstein HA, Sokol JM, Belcikova M, Wayne AS, Zuckerman KS, Sokal L, Castleberry RP, Emanuel PD: Clonality in juvenile chronic myelogenous leukemia. *Blood* 85:21, 1995

48. Fialkow PJ, Denman AM, Jacobson RJ, Lowenthal MN: Chronic myelocytic leukemia: origin of some lymphocytes from leukemic stem cells. *Journal of Clinical Investigation* 62:815, 1978

49. Bernheim A, Berger R, Prud'Homme JL, labaume S, Bussel A, Barot-Ciorbaru R: Philadelphia chromosome positive blood B lymphocytes in chronic myeloid leukemia. *Leukemia Research* 5:331, 1981

50. Lawrence HJ, Broudy VC, Magenis RE, Olson S, Tomar D, Barton S, Fitchen JH, Bagby GC: Cytogenetic evidence for involvement of B lymphocytes in acquired sideroblastic anemia. *Blood* 70:1003, 1987

51. White NJ, Nacheva E, Asimakopoulos FA, Bloxham D, Paul B, Green AR: Deletion of chromosome 20q in myelodysplasia can occur in a multipotent precursor of both myeloid cells and B cells. *Blood* 83:2809, 1994

52. Kere J, Ruutu U, Lahtinen R, de la Chapelle A: Monosomy 7 in granulocytes and monocytes in myelodysplastic syndrome. *New England Journal of Medicine* 316:499, 1987

53. Kroef MJPL, Fibbe WE, Mout R, Janssen RPM, Haak HL, Wessels JW, Van Kamp H, Willemze R, Landegent JE: Myeloid but not lymphoid cells carry the 5q deletiuon: polymerase chain reaction analysis of loss of heterozygosity using mini-repeat sequences on highly purified cell fractions. *Blood* 81:1849, 1993

54. Emanuel PD, Bates LJ, Castleberry RP, Gualtieri RJ, Zuckerman KS: Seletive hypersensitivity to granulocyte-macrophage colony stimulating factor by juvenile chronic myeloid leukemia hematopoietic progenitors. *Blood* 77:925, 1991

55. Freedman MH, Cohen A, Grunberger T, Bunin N, Luddy RE, Saunders EF, Shahidi N, Lau A, Estrov Z: Central role of tumor necrosis factor, GM-CSF, and interleukin 1 in the pathogenesis of juvenile chronic myelogenous leukemia. *British Journal of Haemotology* 80:40, 1992

56. Papayannopoulou T, Nakamoto B, Anagnou NP, Chui D, Dow L, Sanders J: Expression of embryonic globins by erythroid cells in juvenile chronic myelocytic leukemia. *Blood* 77:2569, 1991

57. Privitera E, Schiro' R, Longoni D, Ronchi A, Rambaldi A, Bernasconi S, Ottolenghi S, G. M, Biondi A: Constitutive expression of GATA-1, EPOR, α-globin, and γ-globin genes in myeloid clonogenic cells from juvenile chronic myelocytic leukemia. *Blood* 86:323, 1995

58. Pendergast AM, Quilliam LA, Cripe LD, Bassing CH, Zonghan D, Nanxin L, Batzer A, Rabun KM, Der CJ, Schlessinger D, Gishizky ML: BCR-ABL-Induced oncogenesis is mediated by direct interaction with the SH2 domain of the GRB-2 adaptor protein. *Cell* 75:175, 1993

59. Druker B, Okuda K, Matulonis U, Salgia R, Roberts T, Griffin JD: Tyrosine phosphorylation of RasGAP and associated proteins in chronic myelogenous leukemia cell lines. *Blood* 79:2215, 1992

60. Raitano AB, Halpern JR, Hambuch TM, Sawyers CL: The Bcr-Abl leukemia oncogene activates Jun kinase and requires Jun for transformation. *Proceedings of the National Academy of Sciences* USA 92:11746, 1995

61. Sawyers CL, McLaughlin J, Witte ON: Genetic requirement for Ras in the transformation of fibroblasts and hematopoietic cells by the Bcr-Abl oncogene. *Journal of Experimental Medicine* 18:307, 1995

62. Kaneko Y, Maseki N, Sakuri M, Shibuya A, Shinohara T, Fujimoto T, Kanno H, Nishikawa A: Chromosome patterns in juvenile chronic myelogenous leukemia, myelodysplastic syndrome, and acute leukemia associated with neurofibromatosis. *Leukemia* 3:36, 1989

63. Luna-Fineman S, Shannon K, Lange BJ: Childhood monosomy 7: Epidemiology, biology and mechanistic implications. *Blood* 85:1985, 1995

65. Jacks T, Shih S, Schmitt EM, Bronson RT, Bernards A, Weinberg RA: Tumorigenic and developmental consequences of a targeted *Nf1* mutation in the mouse. *Nature Genetics* 7:353, 1994

65. Hall JG: Genomic imprinting: review and relevance to human diseases. *American Journal of Human Genetics* 46:857, 1990

66. Sapienza C: Genome imprinting, cellular mosaicism and carcinogenesis. *Molecular Carcinogenesis* 3:118, 1990


UC
San Francisco
LIBRARY