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13. ABSTRACT (Maximum 200 Words)

This final report presents research data from studies performed during the initial 9 month period of a 3 year award from the U.S. Army CML research program. In the fall of 2003, the investigators were notified that an R01 proposal on the same subject was approved for funding by the National Cancer Institute. After consultation with Army CML program personnel, we voluntarily relinquished this award so that the unused funds could be invested in another research project relevant to improving the care of patients afflicted with CML. The subject of this grant in the SHP-2 protein tyrosine phosphatase, which is mutated in certain chronic myeloid malignancies. We report substantial progress toward accomplishing the goals of this proposal, which include: (1) screening JMML, CMML, and CML samples for mutations in the *PTPN11* gene, which encodes SHP-2; (2) to introduce mutant *PTPN11* alleles into cultured cell lines and primary murine hematopoietic cells to investigate modulation of signal transduction and cell growth *in vitro* and *in vivo;* and, (3) to generate mice carrying an inducible mutant allele of *PTPN11* that can be expressed in hematopoietic cells.

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INTRODUCTION

Of all human cancers, the molecular pathogenesis of chronic myeloid leukemia (CML) is perhaps the best understood. This knowledge has led to the extraordinary development of imatinib as an effective targeted therapeutic. However, many aspects of CML biology remain mysterious. The critical cellular targets of Bcr-Abl-induced transformation remain elusive. This is a central issue, as understanding the proteins that are responsible for the oncogenic effects of Bcr-Abl will permit the development of new therapies that might overcome primary and acquired resistance to imatinib. The events that induce progression of chronic phase to blast crisis also remain largely unknown.

CML shares clinical and biologic features with chronic myelomonocytic leukemia (CMML) and juvenile myelomonocytic leukemia (JMML). Previous studies of these disorders have illuminated aberrant regulation of biochemical pathways that have also been implicated in the pathogenesis of CML. SHP-2 is a non-receptor tyrosine phosphatase that is a known target of the Bcr-Abl tyrosine kinase and may play a role in activating Ras and other downstream targets. This phosphatase associates with activated growth factor receptors in hematopoietic cells and modulates activation of Ras signal transduction. Recently, mutations in the *PTPN11* gene, which encodes SHP-2, were shown to cause Noonan Syndrome (NS). NS is an autosomal dominant genetic disorder that is associated with facial, cardiac, and skeletal abnormalities. In addition, some children with NS have been reported with CMML and JMML. We have discovered mutations in *PTNP11* in patients with JMML and CMML who do not have NS, and we hypothesize that aberrant activation of this protein may contribute to the abnormal growth of myeloid cells in CML, JMML, and CMML. If this is true, SHP-2 would represent a novel target for drug discovery. The overall goal of this project is to understand how mutations in *PTNP11* alter the function of SHP-2 and how this, in turn, contributes to myeloid leukemia. We are addressing this problem through the technical objective (aims) listed below.

(1) We hypothesize that a proportion of JMML and CMML bone marrow specimens will demonstrate mutations in *PTPN11,* which will be present in the germline of children with stigmata of NS and will be acquired in other patients. Based on the idea that SHP-2, Ras, and neurofibromin are components of the same signaling pathway, we further hypothesize that *PTPN11, RAS,* and *NF1* mutations will exist in mutually exclusive subsets of cases. After we determine the frequency and types of *PTPN11* mutations in JMML and CMML specimens, we will investigate CML bone marrows to test the idea that SHP-2 activation by Bcr-Abl abrogates the requirement for mutations in *PTPN11* in CML.

(2) We hypothesize that mutant *PTPN11* alleles identified in patients with NS and in MPD encode activated SHP-2 molecules that activate Ras and its effector pathways, and that mutant proteins deregulate the growth of cultured cell lines and of primary murine hematopoietic cells. We will use a combination of *in vitro* and *in vivo* approaches to test this hypothesis.

(3) Based on data from studies performed under aims 1 and 2, we will generate a line of mice in which expression of a mutant allele of *PTPN11* is under the control of a tetracycline-

inducible promoter. We hypothesize that these mice will develop MPD that will be dependent upon expression of the transgene.

BODY

Background

Chronic Leukemias Associated with Excessive Production of Myeloid Lineage Cells. CML, CMML, and JMML are characterized by malignant transformation in the stem cell compartment with clonal proliferation of progeny that retain the capacity to differentiate into lymphocytes, erythrocytes, megakaryocytes, monocytes, and neutrophils. Prominent over-production of immature and mature myeloid cells is a feature of all three disorders with splenic infiltration, and splenic enlargement is observed in many patients. CML has been classified as a myeloproliferative syndrome as the cellular morphology is typically normal at diagnosis. The World Health Organization has recently proposed that CMML and JMML be assigned to a mixed category of myelodysplastic/myeloproliferative diseases (1). Beyond nomenclature, this reassessment is logical as molecular investigation of CML, CMML, and JMML have uncovered a number of common pathologic and clinical features. Laboratory investigations of each of these myeloproliferative disorders (MPDs) have enhanced our understanding of the others, and have suggested testable mechanistic hypothesis regarding the genetic and biochemical pathways that are deregulated in myeloid leukemia.

CMML and JMML. CMML is a clonal myeloid malignancy with considerable clinical heterogeneity, which primarily affects elderly individuals and is characterized by a variable degree of myeloid proliferation, monocytosis, and myelodysplasia (2, 3). *RAS* mutations are the most common molecular lesion identified in CMML. When CMML cells are plated in methylcellulose cultures containing variable concentrations of cytokines, a hypersensitive pattern of colony forming unit-granulocyte macrophage (CFU-GM) growth is detected in many cases (4). JMML is a relentless myeloid malignancy of young children characterized by over-production of myeloid lineage cells that infiltrate hematopoietic and non-hematopoietic tissues (5, 6). As in CMML, *RAS* mutations are common in JMML. The incidence of JMML is increased 200-500 fold in children with NF1 (7); this association between NF1 and JMML is intriguing because *NF1* encodes neurofibromin, a GTPase activating protein (GAP) that negatively regulates Ras output by accelerating GTP hydrolysis (8, 9). JMML cells selectively form abnormal numbers of CFU-GM colonies in methylcellulose cultures containing low concentrations of the growth factor granulocyte-macrophage colony stimulating factor GM-CSF (10). Strains of *Nf1* mutant mice provide an animal model of JMML (11, 12). Homozygous *Nf1*-deficient fetal hematopoietic cells are hypersensitive to GM-CSF *in vitro*, and irradiated recipients transplanted with these cells develop MPD (12-14). We exploited the interferon-inducible *Mx1-Cre* transgene and a conditional *Nf1* mutant allele to engineer a new model of MPD (15).

Ras Signaling and GTPase Activating Proteins (GAPs). Ras proteins modulate cell fates by acting as molecular switches in signal transduction (6, 16, 17). Ras output is tightly regulated by cycling between an active guanosine triphosphate (GTP)-bound state (Ras-GTP) and an inactive guanosine diphosphate (GDP)-bound state (Ras-GDP). Ras activation is an essential component

of proliferative responses to a variety of extracellular stimuli including most hematopoietic growth factors. Phosphorylation of tyrosine residues on many activated cytokine receptors creates docking sites for adapter molecules such as Shc and for effector molecules including the p85 regulatory subunit of phosphoinositide-3-OH kinase (PI3K), and SHP-2. Subsequent recruitment of the Grb2 and Gab2 signal relay proteins and the Sos guanine nucloetide exchange factor by phosphorylated Shc represents a major mechanism of Ras activation in hematopoietic cells. Ras-GTP, in turn, signals through a number of downstream effectors such as extracellular related kinase (ERK) and PI3K, which modulate proliferation, survival, and differentiation.

Hyperactive Ras in Myeloid Malignancies. Point mutations of *RAS* genes at codons 12, 13, and 61 are the most common oncogenic lesions found in human cancer cells and are frequently detected in myeloid malignancies. Each of these residues participates in GTP binding and amino acid substitutions constitutively activate Ras signaling by increasing intracellular levels of Ras-GTP. *RAS* mutations occur in 10-40% of myeloid malignancies, with mutations especially prevalent in CMML (3, 18). Other leukemia-associated genetic lesions deregulate Ras signaling. For example, internal tandem duplications and point mutations of *FLT3* are detected in ~25% of human AML patients; biochemical data implicate deregulated Ras/Raf/ERK signaling as an important consequence of these mutations (19). In CML, Bcr-Abl stimulates guanine nucleotide exchange activity through a direct interaction with the Grb2 adaptor, and dominant negative *RAS* alleles block *BCR-ABL*-induced transformation (20, 21). We have shown that *NF1* results in hyperactive Ras in myeloid malignancies from NF1 patients (13, 22, 23). Importantly, *RAS* mutations have not been detected in the leukemia specimens from children with NF1 and are rare in both CML and in AML samples that contain *FLT3* mutations (19, 24, 25). Taken together, these data argue strongly that hyperactive Ras is a common pathogenic feature of human MPD that may be induced by different genetic mechanisms.

SHP-2 Modulates Signaling from Activated Receptors. SHP-2 is a widely expressed cytosolic non-receptor tyrosine phosphatase encoded by the human *PTPN11* gene. The protein includes two src homology 2 (SH2) domains (termed N-SH2 and C-SH2) and a catalytic protein tyrosine phosphatase (PTP) domain (Fig. 1). Analysis of the crystal structure of SHP-2 and experiments using phosphotyrosine peptides revealed that the N-SH2 domain directly blocks the active site of the PTP domain, thereby rendering SHP-2 catalytically inactive (26). The SHP-2 PTPase becomes activated when the SH-2 domain binds an appropriate tyrosine phosphorylated ligand; this results in a conformational shift that permits the active site to interact productively with target molecules. In mammalian cells, SHP-2 participates in signal transduction downstream of growth factor receptors to regulate multiple cellular responses including proliferation, differentiation, and migration (27). SHP-2 is a component of signaling pathways initiated by ligands such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and insulin-like growth factor-1 (IGF-1) in non-hematopoietic cells. The protein becomes rapidly tyrosine phosphorylated upon ligand binding and can bind to docking sites on some activated receptors (28, 29). SHP-2 also associates with various adapter and signal relay proteins such as Grb2, FRS-2, IRS-1, and Gab1. Most often SHP-2 plays a positive role in transducing signals from activated receptors, which is mediated, at least in part, through the Ras/Raf/ERK cascade in many cell types (30). While extensive data place SHP-2 upstream of Ras as a positive effector of ERK signaling, observations in Drosophila and mammalian cells also support a more complex

role in signal transduction that includes functions either parallel to or downstream of Ras-GTP (27, 31).

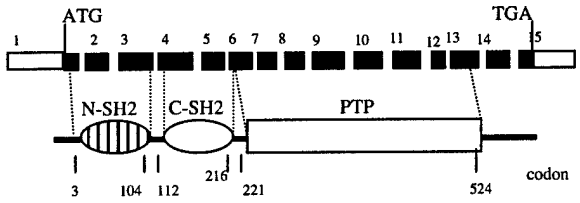

Figure 1. *PTPN11* organization and SHP-2 domain structure. Taken from Tartaglia *et al., Nature Genetics* **29**, 465-468 (2001).

SHP-2 in Hematopoietic Cells. *Ptpn11* is the murine homolog of *PTPN11*. Inactivation of *Ptpn11* has profound effects on the developing hematopoietic system (32-34). Homozygous *Ptpn11* mutant yolk sacs display a marked reduction in the numbers of hematopoietic colony forming cells. SHP-2 is expressed at high levels in hematopoietic cells and undergoes rapid tyrosine phosphorylation upon activation of the c-kit, interleukin 3 (IL-3), GM-CSF, and erythropoietin receptors (27, 35, 36). When the pro-B cell line Ba/F3 is stimulated with IL-3, SHP-2 associates with the signal relay molecule Gab2 (31). This interaction, which involves almost all of the Gab2 found in Ba/F3 cells but only ~10% of total SHP-2, is important for cytokine-induced c-fos promoter activation and Elk1 and STAT5-mediated transactivation. The MEK inhibitor PD98059 blocks c-fos activation in this system, demonstrating an essential role for the Ras/Raf/ERK pathway. However, a mutant Gab2 peptide that retained the ability to bind the p85 subunit of PI3K and Shc but could not associate with SHP-2 surprisingly enhanced ERK activation. By contrast, a SHP-2 mutant with defective phosphatase activity blocked the ERK response to IL-3. These data implicate SHP-2 as functioning at multiple points in cytokine signaling both upstream and parallel to Ras/Raf/ERK (31). Further work has characterized the role of Gab2 and SHP-2 in signaling from the common β chain (β^c) of the GM-CSF, IL-3, and IL-5 receptors (see Fig. 2). Gab2 is constitutively associated with the Grb2 adapter in unstimulated Ba/F3 cells engineered to respond to GM-CSF. This complex is rapidly recruited to phosphorylated Shc on Tyr-577 of activated β^c, recruits the p85 subunit of PI3K, and induces downstream activation of protein kinase B (PKB, also known as Akt). Interestingly, SHP-2 also binds directly to the activated GM-CSF receptor at Tyr-612 and at Tyr-695, which results in phosphorylation of Gab2. It is unknown if these interactions also stimulate downstream signaling cascades through Gab2-independent interactions. Most recently, co-immunoprecipitation experiments in Ba/F3 cells engineered to express Bcr-Abl showed that SHP-2, Gab2, and the p85 subunit of PI3K associate with Tyr-177 of the Bcr domain, which is a Grb2 binding site on the fusion protein (37). Remarkably, primary hematopoietic cells from Gab2 mutant mice were resistant to transformation by retroviruses encoding Bcr-Abl; this was associated with substantial reductions in ERK and PKB activation. These data raise the intriguing possibility that the association of activated SHP-2 with Bcr-Abl contributes to CML, perhaps through effects on Ras/Raf/ERK signaling (37). In work performed under this award, we have shown that oncogenic SHP-2 mutations contribute to myeloid leukemogenesis.

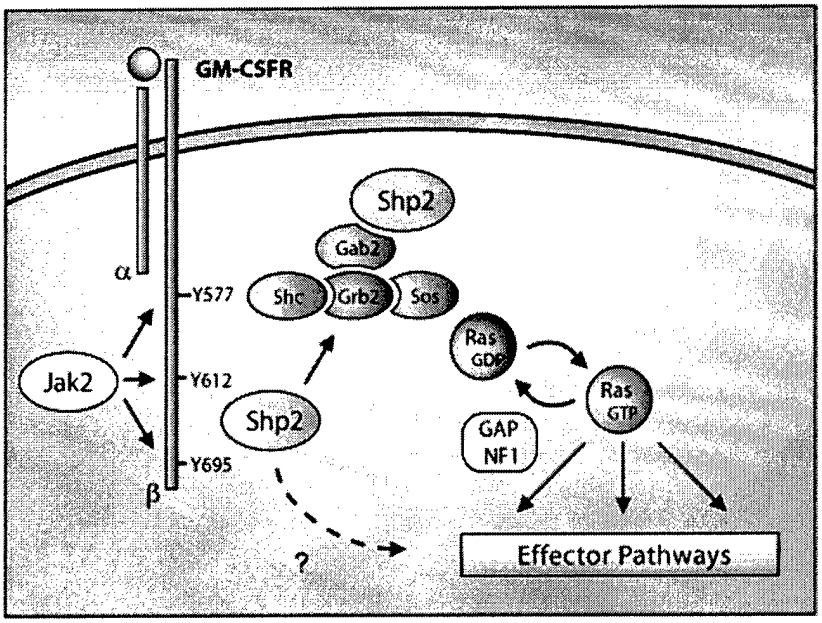

Figure 2. Overview of SHP-2 in signlaing from the GM-CSF receptor to Ras. Upon lignad binding, Jak2 phosphorylates 8 tyrosine residues on βc including 3 (Y577, Y612, and Y695) that have been implicated as mediating signaling via SHP-2. Y577 is a major binding site for Shc, and this recruits Gab2, Grb2, and SHP-2, which activate Ras via Sos. SHP-2 can also bond directly to Y612 and Y695, and may activate the Grb-Gab-2 complex independent of Shc (solid line) and/or signal through effector cascades that are downstream or parallel to Ras-GTP (dotted line).

PTPN11 and Noonan Syndrome. Missense mutations in *PTPN11* were recently shown to cause Noonan syndrome (NS), a developmental disorder characterized by cardiac defects, facial dysmorphism, and skeletal malformations (38, 39). Children with NS show a spectrum of hematologic abnormalities including isolated monocytosis, myeloid disorders with features of CMML that remit spontaneously and, rarely, JMML (40-42). Bone marrow cells from NS patients with JMML show characteristic GM-CSF hypersensitivity in methylcellulose cultures (41). These observations, data implicating hyperactive Ras in the pathogenesis of JMML, and the role of SHP-2 in relaying signals from hematopoietic growth factor receptors to Ras identify *PTPN11* as an excellent candidate gene that might be mutated in cases of JMML without abnormalities in *RAS* or *NF1* and other myeloid maligncies. We propose a series of experiments to further elucidate the role of *PTPN11* and SHP-2 in myeloid leukemogenesis.

Progress Report

Technical Objective (Aim) 1: PTPN11 Mutations in JMML, CMML, and CML

Our initial genetic and biochemical experiments to ascertain the incidence of *PTPN11* mutations in leukemia samples and to perform biochemical studies of leukemia-associated SHP-2 proteins were published recently (43). Reprints of this paper are attached to this report and the major findings are summarized here and in Technical Objective 2. Based on case reports of JMML in NS patients, we first investigated 51 JMML specimens for *PTPN11* mutations including two from children with a clinical diagnosis of NS. These studies uncovered missense mutations in 16 of 49 JMML samples from patients without NS. Fifteen of 16 *PTPN11* mutations detected in sporadic cases of JMML occurred in exon 3, which encodes a segment of the N-SH2 domain. The only exception was an exon 4 mutation that we identified in a specimen

from a 2-month infant. Both of the JMML specimens from children known to have NS demonstrated different *PTPN11* mutations from the non-NS patients, including one substitution in exon 13.

The 49 JMML specimens from patients without NS were divided into 3 groups: (1) samples from patients with a clinical diagnosis of NF1 or an *NF1* mutation, (2) samples with *RAS* (*KRAS* or *NRAS* mutations), or (3) all other JMML samples. When these groups were compared, we observed a statistically significant difference in the frequency of *PTPN11* mutations in group 3 versus groups 1 and 2 . These data provide genetic evidence that mutant SHP-2 proteins contribute to leukemogenesis through a Ras-dependent mechanism. We noticed absence of the normal *PTPN11* allele on the DHPLC tracing of a JMML specimen with a mutation at nucleotide 215. This C-to-T mutation ablates a Bgl I cleavage site in the normal sequence and creates a new Mae III site. Digestion of PCR products amplified from exon 3 confirmed that the normal allele was absent in this case. To address if loss of the normal allele exists in other cases of JMML with *PTPN11* mutations, we performed PCR amplification followed by allele-specific cleavage of 15 additional cases. All of these leukemias retained the normal *PTPN11* allele.

We next surveyed CMML, CML, AML, and MDS specimens for *PTPN11* mutations. Mutations were detected in 7 of these samples (11%), including 1 of 4 from patients with CMML, 0 of 11 with CML, 3 of 28 with AML, 2 of 7 with MDS, and in 1 of 10 in patients with theraprelated MDS or AML. Each mutation was in exon 3 and many were identical to those found in JMML patients. The absence of *PTPN11* mutations in CML is consistent with genetic and biochemical data implicating Ras as a downstream target of Bcr-Abl in myeloid leukemogenesis (44, 45).

Based in the clinical and biologic similarities between JMML and CMML, we recently extended our analysis to interrogate a large series of specimens from adults with CMML and other types of MDS for *PTPN11* mutations. Surprisingly, <5% of these samples showed mutations. We also screened 278 AML specimens from children who were entered onto two prospective clinical trials conducted by the Children's Cancer Group, and detected 11 *PTPN11* mutations. Patients with *PTPN11* mutations were more likely to be boys with high white blood cell counts at diagnosis with FAB subtype M5 AML. A manuscript describing the CMML/MDS data is in preparation. A paper summarizing our findings in pediatric AML was recently submitted for publication and is provided with this report.

Technical Objective (Aim) 2: Biochemical and Functional Analysis of Mutant SHP-2 Proteins.

Ba/F3 cells have been used to investigate SHP-2 activation in hematopoietic cells (46-48) and to interrogate the functional consequences of leukemia-associated mutant proteins (49-53). We constructed retroviral vectors in the murine stem cell virus (MSCV) backbone (54) in order to express the leukemia-associated mutant D61Y and E76K SHP-2 proteins identified in this pro-B cell line, which is dependent upon IL-3 for survival and proliferation. The data from these experiments are included in the attached article by Loh, *et al.* (55). Briefly, Ba/F3 cells were infected with retroviral vectors that we engineered to co-express wild-type or mutant SHP-2 with a puromycin resistance gene, then cultured with IL-3 and puromycin. This infection/selection procedure was performed on three independent occasions to insure that the biologic effects of expressing mutant SHP-2 proteins were reproducible. After 4 days, the transduced Ba/F3 cells were transferred to medium without IL-3 to assess the effects of expressing wild-type or mutant

SHP-2 on survival and proliferation. At this time, cells were also collected to measure SHP-2 expression and to assay Ras effector cascades. Wild-type and mutant SHP-2 proteins were expressed at similar levels that were higher than in parental Ba/F3 cells. We investigated ERK and Akt phosphorylation in cells that were deprived of serum and IL-3 for 6 hours, then stimulated with IL-3. Surprisingly, we did not observe increased levels of phosphorylated ERK or Akt in resting or IL-3-stimulated Ba/F3 cells that expressed either mutant SHP-2 protein. Signal transduction experiments were performed under a variety of experimental conditions that included varying the concentration of IL-3 and the time course with similar results. We also assessed the effects of wild-type and mutant SHP-2 proteins on the survival and growth of transduced Ba/F3 cells after IL-3 withdrawal. Under these conditions, expression of the E76K mutant consistently enhanced the survival of Ba/F3 cells. We also observed subtle, but reproducible, effects of the D61Y SHP-2 protein. Importantly, Ba/F3 cells transduced with mutant SHP-2 proteins did not expand during the 2-3 week culture period, but sustained higher numbers of viable cells (55). These data infer that a major effect of leukemia-associated mutant SHP-2 proteins is to enhance the survival of hematopoietic cells that are deprived of exogenous growth factors.

We also infected murine fetal liver cells and bone marrow cells from mice that had been treated with 5-fluoruracil with our MSCV vectors to ascertain the effects of expressing the E76K and D61Y mutations in primary hematopoietic cells. Control cells were infected in parallel with an empty MSCV vector or with a vector encoding the wild-type SHP-2 protein. Both mutant SHP-2 proteins had dramatic effects on the growth of myeloid colony forming unit granulocyte-macrophage (CFU-GM) progenitors. Fetal liver and bone marrow cells that expressed the E76K or D61Y mutations, but not cells expressing wild-type SHP-2, formed CFU-GM in methylcellulose medium without added growth factors and demonstrated a hypersensitive pattern of progenitor colony growth in response to GM-CSF or interleukin-3 (Fig. 3).

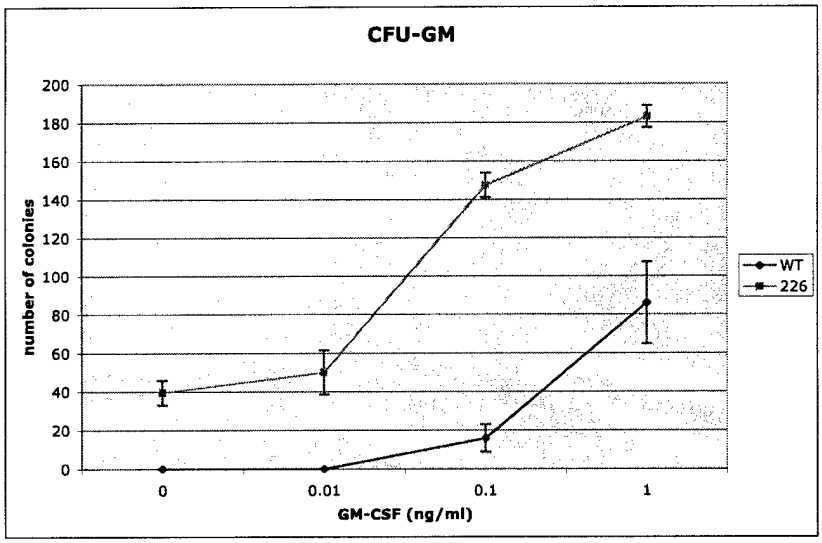

Figure 3. CFU-GM colony growth over a range of GM-CSF concentrations in mouse fetal liver cells engineered to express either wild-type SHP-2 (WT) or the E76K mutant, which results from a mutation at nucleotide 226. Cells expressing mutant SHP-2 show growth factor-independent CFU-GM formation and a hypersensitive pattern of growth with exogenous GM-CSF.

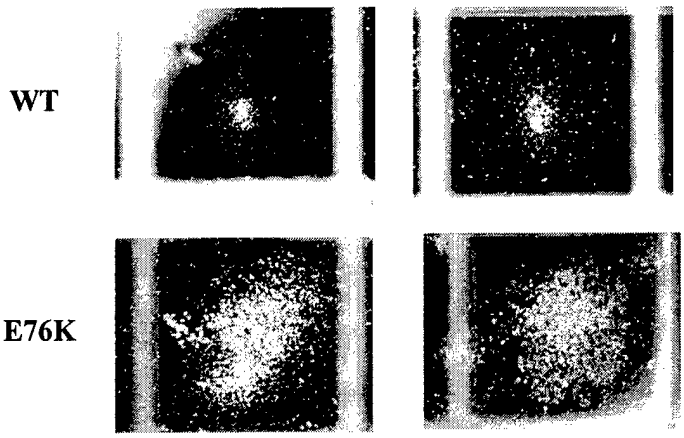

Figure 4. Morphology of CFU-GM colonies grown from fetal liver cells expressing either wild-type or mutant SHP-2 in 1 ng/mL or GF-CSF. The mutant colonies are much largers and contain many more cells.

The E76K mutant protein, which promotes Ba/F3 survival to a greater effect than the D61Y substitution and has a higher intrinsic phosphatase activity (56), had a more potent effect on CFU-GM growth. In addition to these effects on colony numbers, CFU-GM derived from cells expressing the E76K or D61Y mutant SHP-2 proteins were much larger than normal colonies (Fig. 4). These data demonstrate that mutant SHP-2 proteins perturb the proliferation of primary myeloid progenitors *in vitro* and render these cells hypersensitive to hematopoietic growth factors. We have injected cells transduced with wild-type or mutant SHP-2 vectors into irradiated mice and are observing recipient animals for evidence of myeloid disease.

Technical Objective (Aim) 3: Production and Characterization of Ptpn11 Transgenic Mice

We considered two possible strategies for generating a strain of transgenic mice in which we can induce expression of a mutant *PTPN11* allele *in vivo*. The first of these is a tetracycline inducible system, and the second is to develop transgenic strains of mice in which wild-type or mutant alleles of *PTPN11* are introduced with a bacterial artificial chromosome (BAC) clone. Based on the fact that transgenes introduced within BACs are more likely to be expressed at physiologic levels, recent technical advances in manipulating these vectors, and the comments of the reviewers of our proposal, we have been pursuing the latter strategy. We also considered introducing the mutant *PTPN11* alleles into the endogenous locus with a "knock in" approach using embryonic stem cells. However, we felt that the BAC transgenic approach would offer a system for efficiently comparing different mutant alleles, which is more challenging using the knock in approach.

Using the National Center for Biotechnology website, www.ncbi.nlm.nih.gov, and sequences available from the Ensembl website, www.ensembl.org/Mus_musculus, we searched for mouse BAC clones containing *Ptpn11*. We obtained 2 clones from the Children's Hospital Oakland Research Institute BAC/PAC resource, www.chori.org/BACPAC/mapped-clones.htm, which include the entire *Ptpn11* locus as well as 5' upstream and 3' downstream sequences. The 2 clones are: (1) RP23-257E17 (180,681 bp), which includes approximately 46 kb 5' and 77 kb 3' to the *Ptpn11* coding region, and, (2) RP24-535J15 (117,992 bp), which includes 55 kb 7 and 5 kb 3 to *Ptpn11*. We first performed PCR experiments, which demonstrated that both BAC clones contain the entire *Ptpn11* locus (data not shown). Further characterization included pulse

field gel electrophoresis analysis comparing the restriction digestion patterns as well as Southern blots to ensure the integrity of the BACs (data not shown). Based on these studies we selected clone RP23-27E17 for the future experiments as the complete sequence of this BAC is available.

Recombineering refers to utilizing efficient homologous recombination systems that have been recently developed for use in *E. coli* for chromosome engineering by various methods (reviewed (57). We chose to use the phage encoded recombination system, which is 10-100 fold more efficient than the previous recombination systems available in *E. coli*. The λ phage contains a homologous recombination system termed Red. Red recombination requires two genes, red α (exo) and red β (bet), as well as the phage-encoded Gam gene which inhibits the RecBC activity of the host cell. Because the phage-encoded enzymes are induced at 42 degrees, this efficient recombination system can be briefly induced to mutagenize the *Ptpn11*BAC and then inhibited to allow the BAC to remain stable in the host cell.

Our initial experimental strategy involved inserting a SacB gene, which allows negative selection in sucrose and a neomycin resistance gene into exon 3 of *Ptpn11* by homologous recombination. This modification was accomplished without difficulty (Fig. 5A, B). We next attempted to generate specific mutations (D61Y, T73I, E76K, and a silent wild-type polymorphism) by introducing synthesized oligonucleotides, followed by screening transformants that have lost the SacB gene and integrated the altered oligonucleotide containing the specific mutation without leaving any selectable marker sequence. However, this step was initially unsuccessful as recombinants that were now SacB resistant were found to favor large deletions versus simple replacement of SacB-Neo with the mutation containing oligonucleotides. This result suggested that the homologous recombination with oligonucleotides were less efficient than gross deletions in the locus. We postulated that the deletions were caused by repetitive elements that are present throughout the mouse genome as well as the SacB sequences, which are often present in the BAC vector. Our attempts to increase the efficiency by increasing the size of homology, using sense or anti-sense nucleotides were unsuccessful. We therefore decided to remove the SacB sequences in the vector arm in an attempt to further increase the efficiency of the desired recombination. Using hybrid oligonucleotides lateral to the SacB sequences with Ampicillin sequences, a recombination cassette to replace the Sac B sequences with Ampicillin was made and *Ptpn11* BACs lacking the additional SacB sequences in the vector were generated. After making this modification, we were abel to generate an allele series of *Ptpn11* mutant BACs, which were engineered to contain only the desired nucleotide changes (Fig. 5C). These altered BACs have now been sequenced to verify the mutations and we have succeeded in generating transgenic mice that carry the wild-type BAC (Fig. 5D). We produced two transgenic lines carrying this BAC out of 17 pups screened, which is consistent with the expected integration rate. However, none of the 40 pups from the mutant BAC carrying the JMML mutation, D61Y, carried the transgene. We hypothesize that this is the consequence of over-expression of the mutant Shp-2 protein being deleterious to development.

Due to our concerns that constitutive expression of leukemia-associated *Ptpn11* mutations will be lethal in embryonic life, we plan to overcome this problem by further modifying our existing BAC clones to introduce a *loxP-stop-loxP* (LSL) element upstream of the promoter. We are exploiting the same LSL cassette that the Jacks lab used to create a conditional mutant allele of *Kras*. We have recently shown that the interferon-inducible *Mx1-Cre* transgene efficiently activates expression of this allele, which results in a fatal MPD (58). Based on these data, we will intercross founder *Ptpn11* mice with *Mx1-Cre* animals, and will inject the compound mutant offspring with polyinosinic-polycytidilic acid (pIpC) to activate Cre

recombinase expression in hematopoeitic cells. These mice will be characterized for evidence of MPD, as described previously (15, 58).

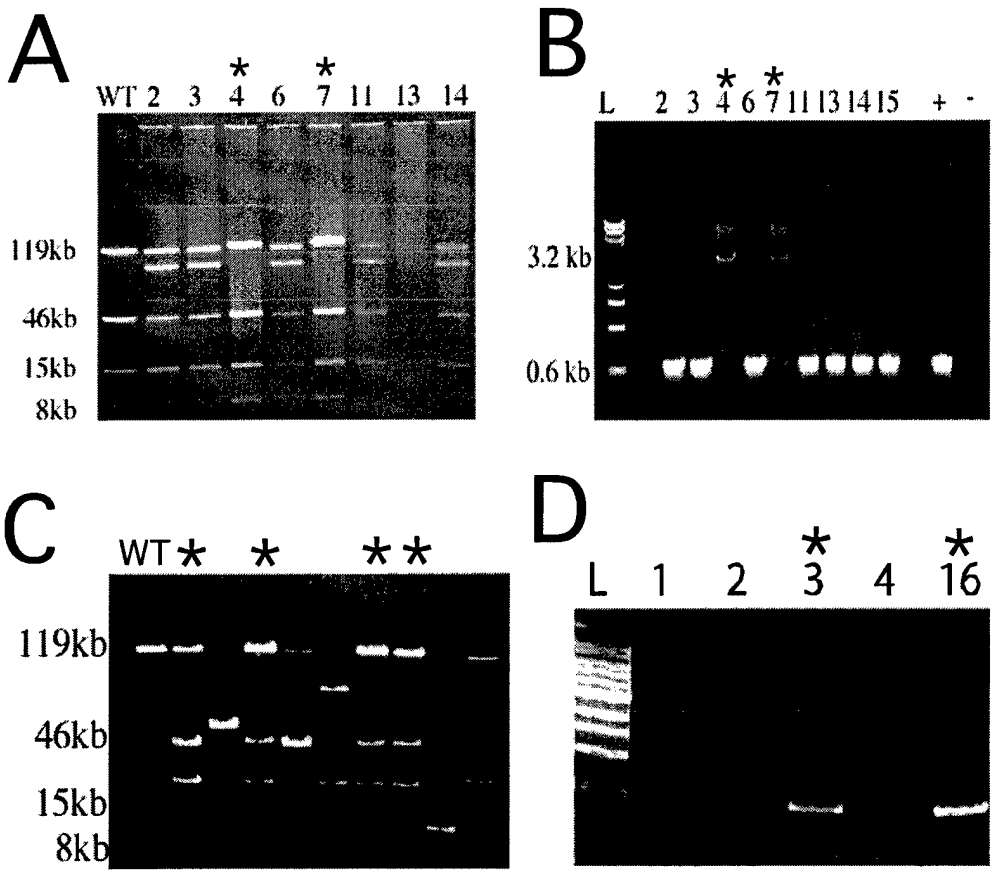

Figure 5. BAC Clones Containing *Ptpn11* Mutations and Production of Transgenic Mice. Panel A. NotI-digested pulse field gel electrophoresis (PFGE) of bacterial clones after electroporation and selection in kanamycin. The targeting amplicon contained the SacB-Neo cassette flanked by exon 3 sequences allowing the precise targeting into exon 3. Note that samples 4 and 7 contain the correctly targeted BAC whereas the other lanes show BACs with undesired deletions. Panel B. PCR using intronic primers, which flank exon 3 of the *Ptpn11* gene. Note that only clones 4 and 7 contain the 3.2 kb amplicon, which shows that the sacB-Neo cassette has been integrated correctly integrated. Panel C. PFGE analysis of bacterial DNA samples after electroporation with oligonucleotides containing specific nucleotide changes corresponding to the desired *Ptpn11* mutations. After electroporation, the bacteria are grown in 5% sucrose, which provides negative selection against sacB gene. Resistant clones have either resulted gross deletions of exon 3 containing the sacB gene or have replaced the sacB gene with the oligonucleotide containing the desired mutations. Sequencing confirmed the correct mutations. Panel D. PCR amplification of tail DNA of pups generated after injection of wild type *Ptpn11* BAC into fertilized mouse oocytes. Mouse number 3 and 16 contain the transgene. The primers used in this amplification recognize sequences at the end of the BAC and does not amplify the endogenous *Ptpn11* gene.

KEY RESEARCH ACCOMPLISHMENTS

(a) We have defined the spectrum of *PTPN11* mutations in myeloid malignancies including JMML, CMML, AML, and MDS, and have described the relationship of *PTPN11* mutations to mutations in *RAS* and *NF1*.

(b) We have shown expressing leukemia-associated *PTPN11* alleles promotes the survival of Ba/F3 cells and we have investigated Ras signaling in this cell line.

(c) We have shown that expressing leukemia-associated *PTPN11* alleles induces growth factor hypersensitivity and hyperproliferation in methylcellulose cultures of primary myeloid progenitor cells.

(d) We have injected fetal liver and bone marrow cells transduced with leukemia-associated *PTPN11* alleles into irradiated recipient mice and are monitoring these animals for signs of MPD.

(e) We have introduced expressing leukemia-associated *PTPN11* mutations and a silent nucleotide substitution into a mouse BAC vector spanning the mouse Ptpn11 locus and have successfully generated founders for the wild-type (silent) allele.

REPORTABLE OUTCOMES

(a) Research Articles and Reviews

Loh ML, Vattikuti S, Reynolds MG, Carlson EJ, Schubbert S, Lieuw KH, Cheng JW, Stokoe D, Bonifas JM, Curtiss NP, Gotlib J, Meshinchi S, Le Beau MM, Emanuel PD, Shannon KM. Somatic mutations in *PTPN11* implicate the protein tyrosine phosphatase SHP-2 in leukemogenesis. *Blood* 2004; **103:** 2325-2331.

Tartaglia M, Niemeyer CM, Shannon KM, Loh ML. SHP-2 and myeloid malignancies. *Curr Opin Hematol* 2004: 11: 44-50.

Loh ML, Reynolds, MG, Vattikuti S, Gerbing RB, Alonzo TA, Cheng JW, Lee CM, Lange BJ, Meshinchi S. *PTPN11* mutations in pediatric patients with acute myeloid leukemia: a report from the Children's Cancer Group, in review.

(b) Abstracts

Loh ML, Vattikuti S, Schubbert S, Reynolds MG, Carlson E, Lieuw KH, Cheng JW, Lee CM, Stokoe D, Bonifas JM, Curtiss NP, Emanuel PD, Shannon KM. "Somatic mutations in *PTPN11* implicate the protein tyrosine phosphatase SHP-2 in leukemogenesis." *Blood*, 2003,selected for oral presentation at the American Society of Hematology meeting, December 5-9, 2003.

Loh ML, Reynolds MG, Vattikuti S, Cheng JW, Lee CM, Gerbing RB, Alonzo TA, Heerema N, Lange BJ, Meshinchi S. "Incidence and Prognostic Significance of *PTPN11* Mutations in Pediatric Acute Myelogenous Leukemia – Results from Children's Cancer Group Trials 2941 and 2961." *Blood*, 2003, selected for poster presentation at the American Society of Hematology meeting, December 5-9, 2003.

Loh ML, Vattikuti S, Reynolds MG, Cheng JW, Lee CM, Gotlib J, Beran M. "*PTPN11* mutations are rare in adult patients with chronic myelomonocytic leukemia (CMML)." *Blood*, 2003, abstract-publication only for the American Society of Hematology.

(b) Employment and Research Opportunities

This award has provided salary support for technical personnel in each of participating labs.

CONCLUSIONS

During the 9 month period or research support, we have used funds provided by this Program to generate novel genetic, biochemical, and cell biologic data regarding the role of the *PTPN11* gene in myeloid growth control and leukemogenesis. We have also made considerable progress toward the goal of generating relevant animal models. Cases of MPD that are initiated by *PTPN11* mutations are likely to perturb signaling networks that contribute to the pathogenesis of CML, CMML, JMML, and other myeloid malignancies. We will carry this work forward under a new R01 award from the National Cancer Institute.

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PTPN11 Mutations in Pediatric Patients with Acute Myeloid Leukemia: Results from the Children's Cancer Group

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Abstract

The *PTPN11* gene encodes SHP-2, a non-receptor protein tyrosine phosphatase that relays signals from activated growth factor receptors to $p21^{ras}$ (Ras) and other signaling molecules. Somatic *PTPN11* mutations are common in patients with juvenile myelomonocytic leukemia (JMML) and have been reported in some other hematologic malignancies. We analyzed specimens from 278 pediatric patients with acute myelogenous leukemia (AML) who were enrolled on Children's Cancer Group trials 2941 and 2961 for *PTPN11* mutations. Missense mutations of *PTPN11* were detected in 11 (4%) of these samples. None of these patients had mutations in *NRAS*; however one patient had evidence of a *FLT3* alteration. Patients with *PTPN11* mutations were more likely to be boys with French-American-British (FAB) morphology M5 AML who presented with elevated white blood counts. These characteristics identify a subset of pediatric AML with *PTPN11* mutations that share clinical and biologic features with JMML.

Introduction

Acute myeloid leukemia (AML) is characterized by expansion of myeloid blasts with suppression of normal hematopoiesis. Cytogenetic and molecular studies have defined AML as a heterogeneous disease comprised of genetically distinct clonal disorders. Whereas recurring chromosomal translocations are a hallmark of AML, these genetic lesions are thought to cooperate with mutations in genes that encode growth factor receptors and downstream effectors[1]. *FLT3* and *CKIT* encode class III receptor tyrosine kinases that activate Ras and other signaling cascades[2]. Dominant activating mutations in *FLT3*, *CKIT, NRAS* and *KRAS* have been reported in up to 50% of patients with AML[2-4]. Uncovering other mutations that perturb the RTK/Ras signaling pathways could further illuminate the biology of AML, provide new independent prognostic markers, and/or identify new therapeutic targets.

The *PTPN11* gene encodes SHP-2, a non-receptor protein tyrosine phosphatase that relays signals from activated growth factor receptors to Ras and other signaling molecules[5]. Germline mutations in *PTPN11* are a major cause of Noonan syndrome (NS)[6,7]. Children with NS are at increased risk of developing JMML, a relentless myeloproliferative disorder characterized by over-production of myelomonocytic cells that infiltrate spleen, liver, and skin, among other organs[8,9]. Most children with JMML are boys[8,9]. We and others have recently reported that up to 35% of patients with JMML harbor missense *PTPN11* mutations, which are largely exclusive of *RAS* and *NF1* alterations[10,11]. All of these mutations are predicted to activate SHP-2 phosphatase activity by disrupting the inhibitory interaction between the N-SH2 and PTP domains of the protein. Although the biochemical data are inconclusive, genetic studies suggest that the hyperactive *PTPN11* mutations found in JMML contribute to leukemia growth by deregulating the Ras pathway[11,12].

Based on the existence of *NRAS* and *KRAS* mutations in AML and JMML, we reasoned that mutations in *PTPN11* might also occur in both diseases. To address this question, we screened a well-characterized cohort of AML specimens from children enrolled on Children's Cancer Group (CCG)

clinical trials 2941 and 2961 for mutations in *PTPN11* and correlated these data with clinical and molecular data.

Methods

Patients and Therapy. Bone marrow specimens from 298 patients with *de novo* AML registered on protocols CCG 2941 and 2961 were included in this study. A total of 278 specimens had adequate DNA for analysis. The diagnosis of AML was made according to FAB classification and confirmed by central review. The treatments delivered on CCG 2941 and 2961 have been previously detailed[13,14] . The study was approved by the UCSF Committee on Human Research and the CCG Myeloid Biology Committee.

Mutation Detection. DNA was extracted from cryopreserved bone marrow cells using PureGene reagents (Gentra Systems Inc, Minneapolis, MN). Methods for the mutational analysis for *PTPN11*, *NRAS*, and *FLT3 ITD* and *FLT3/ALM* have been previously described[2,7,11,15]. Based on the locations of mutations in NS and JMML, exons 3, 4, 5, 6, 7, 8, 11, 12, and 13 of *PTPN11* were screened.

Statistical Methods. Data from CCG 2941 (through March 2002) and 2961 through (through January 2004) were used to compare patients who had samples analyzed for *PTPN11* mutations with those who did not, and to assess the characteristics of patients with and without*PTPN11* mutaitons. The significance of observed differences was tested using the chi-squared and Fisher exact texts. For continuous data, the Mann-Whitney test was used to compare the medians of distributions. Patients who were lost to follow-up were censored at their last known point of study, with a cutoff of Sept 2001 (CCG 2941) or July 2003 (CCG 2961). Hazard ratio estimates with corresponding 95% confidence intervals (C.I.) of overall survival (OS) and event-free survival (EFS) from study entry and disease-free survival (DFS), defined as the time from achieving remission to marrow relapse or death, were calculated using Cox regression models.

Results and Discussion

To determine whether our study population was representative of the entire CCG-2941/2961 population (n = 988), the clinical characteristics and outcome of the 278 analyzed patients were compared to the 710 patients that were not studied. There were no significant differences with respect to age, sex, race, presenting white count, hemoglobin, platelet count, or FAB morphology. Cytogenetic information was available on 549 cases (56%), including 170 that we analyzed for *PTPN11* mutations. There were no significant differences with respect to the distribution of previously defined good, poor, or standard risk cytogenetics[16], survival , or event-free survival between the study population and the rest of the patient cohort (data not shown).

We identified 11 *PTPN11* mutations in the 278 analyzed patients (4.0%, 95% C.I. 1.7-6.2%), which are listed in Table 1. All of the mutations encode amino acid substitutions within the N-SH2 domain where it interacts with the PTP domain (Table 1). As in JMML, the majority of mutations are located in exon 3 with "hotspots" at codons 72 and 76[10,11].

Clinical characteristics of the study population are summarized in Table 2. There were no differences in diagnostic parameters including the median age, percentage of bone marrow blasts, presence of central nervous system (CNS) disease, hemoglobin, or platelet counts of patients with and without mutations. *PTPN11* mutations were more common in boys, but this was not statistically significant (5.9% vs. 1.6% p= 0.119). Five of the 11 patients (46%) with *PTPN11* mutations had FAB M5 morphology compared to 37/261 (14%) of those without mutations (p =0.016). In addition, there was a trend for patients with *PTPN11* mutations to present with a white blood cell count >100,000 (p=.102). The probabilities of achieving a first clinical remission as well as event-free and overall survival were similar in patients with and without *PTPN11* mutations. However, the power to detect significant differences is diminished by the low frequency of mutations.

These AML samples were also analyzed for *FLT3* and *NRAS* mutations. One of 11 patients with *PTPN11* mutations (9%) had an internal tandem duplication of the *FLT3* receptor gene compared with

17% of the rest of the cohort (p = 0.808). Whereas the overall incidence of *NRAS* mutations was 10% in 266 analyzed samples, none of the 11 specimens with *PTPN11* mutations showed an *NRAS* mutation.

PTPN11 mutations are found in ~35% of JMML samples[10,11]; however, we detected a much lower incidence in *de novo* pediatric AML (4%). Patients with *PTPN11* mutations tend to be boys who present with markedly elevated leukocyte counts and FAB M5 leukemia. Interestingly, the rare cases of AML that evolve from JMML typically show FAB M4 or M5 morphology. The clinical and biologic similarities between JMML and pediatric AMLs with *PTPN11* raise the possibility that both of these myeloid malignancies are initiated by a *PTPN11* mutation. Indeed, genetic analysis of human JMML specimens and the phenotype of *Kras* and *Nf1* mutant mice infer that hyperactive Ras can initiate a myeloproliferative disorder[17,18]. However, the existing data also support a model in which AML is initiated by genetic lesions leading to aberrant transcription that cooperate with mutations that deregulate signal transduction pathways[17,18]. Consistent with this idea, three of the AML patients with *PTPN11* mutations also showed recurrent leukemia-associated cytogenetic abnormalities. Rather than acting as an initiating event in AML, we hypothesize that hyperactive SHP-2 proteins favor the outgrowth of malignant cells, perhaps by perturbing signal transduction from specific cytokine receptors.

The lack of *NRAS* mutations in pediatric AML specimens with *PTPN11* mutations is consistent with the idea that *PTPN11* mutations undermine myeloid growth by deregulating Ras. Similarly, *PTPN11* mutations are largely restricted to JMML specimens without mutations in either *RAS* or *NF1*, which encodes a GTPase activating protein that negatively regulates Ras output. Some, but not all, cultured cell lines engineered to express mutant SHP-2 proteins show hyperactive Ras signaling[10,11,19]. Further studies are required to address how *PTPN11* mutations contribute to specific subsets of AML, and to fully characterize the biochemical consequences of leukemia-associated SHP-2 proteins in hematopoietic cells.

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Table 1. *PTPN11* mutations in pediatric AML

UPN	Age (year)	Sex	FAB	Nucleotide substitution	Amino acid change	ISCN
246	9.5	M	M5	A227G	76E>G	
277	15.9	M	M5	G226A	76E>K	46 XY. +1 der(1;22)(q10;q10), del(11q23)[20]
463	1.3	F	M5	C1505T	502S>L	
485	6.0	M	M5	C215T	72A>V	
497	6.4	M	M5	G226C	76E>Q	
523	1.5	M	M0	G205A	69E>K	50XY, +6, +10, +21, +mar[17]/46, XY[3]
530	13.3	M	M2	C215T	72A>V	46XY, t(8;21)(q22;q22), del(9)(q13q22)[22]
541	5.5	F	M4	G226A	76E>K	46XX[20]
652	9.4	M	M2	T213G	71F>L	
657	14.5	M	M4	C218T	73T>I	
659	2.6	M	M4	A227G	76E>G	

Table 2. Clinical and laboratory characteristics of the analyzed cohort.

Characteristic	*PTPN11* Mutation (N=11)	*PTPN11* No Mutation (N=267)	*p*-value (mutation vs. no mutation)
Median age (years)	6.4	9.7	0.542
Gender			
Male N (%)	9 (82)	144 (54)	0.119
Female N (%)	2 (18)	123 (46)	
Median WBC, x 10^9/L (range)	26.7 (1.6-241)	22.5 (1.0-860)	0.524
< 100,000 (%)	7 (64)	223 (84)	0.102
> 100,000 (%)	4 (36)	44 (16)	
FAB class			0.123
M0, n(%)	1(9)	13(5)	0.447
M1, n(%)	0	43(17)	0.222
M2, n(%)	2(18)	74(28)	0.733
M4, n(%)	3(27)	75(29)	1.00
M5, n(%)	5(46)	37(14)	0.016
M6, n(%)	0	6(2)	1.000
M7, n(%)	0	13(5)	1.000
Cytogenetics			
Good, n(%)	1 (25)	46 (28)	0.697
Poor, n(%)	0	3 (2)	1.000
Standard, n(%)	3(75)	117 (71)	0.317
CR (M1 marrow) (%)	73%	82%	0.421
PR (M2 marrow)(%)	9%	5%	0.427
EFS HR (95% C.I.)	0.86 (0.38-1.94)	1.0	0.716
OS HR (95% C.I.)	0.72 (0.26-1.94)	1.0	0.509

PTPN11

Mignon L. Loh, Shashaank Vattikuti, Suzanne Schubbert, Melissa G. Reynolds, Elaine Carlson, Kenneth H. Lieuw, Jennifer W. Cheng, Connie M. Lee, David Stokoe, Jeannette M. Bonifas, Nicole P. Curtiss, Jason Gotlib, Soheil Meshinchi, Michelle M. Le Beau, Peter D. Emanuel, and Kevin M. Shannon

The *PTPN11* gene encodes SHP-2 (Src homology 2 domain–containing protein tyrosine Phosphatase), a nonreceptor tyrosine protein tyrosine phosphatase (PTPase) that relays signals from activated growth factor receptors to $p21^{Ras}$ (Ras) and other signaling molecules. Mutations in *PTPN11* cause Noonan syndrome (NS), a developmental disorder characterized by cardiac and skeletal defects. NS is also associated with a spectrum of hematologic disorders, including juvenile myelomonocytic leukemia (JMML). To test the hypothesis that *PTPN11* mutations might contribute to myeloid leukemogenesis, we screened the entire coding region for mutations in 51 JMML specimens and in selected exons from 60 patients with other myeloid malignancies. Missense mutations in *PTPN11* were detected in 16 of 49 JMML specimens from patients without NS, but they were less common in other myeloid malignancies. *RAS, NF1,* and *PTPN11* mutations are largely mutually exclusive in JMML, which suggests that mutant SHP-2 proteins deregulate myeloid growth through Ras. However, although Ba/F3 cells engineered to express leukemia-associated SHP-2 proteins cells showed enhanced growth factor–independent survival, biochemical analysis failed to demonstrate hyperactivation of the Ras effectors extracellular-regulated kinase (ERK) or Akt. We conclude that SHP-2 is an important cellular PTPase that is mutated in myeloid malignancies. Further investigation is required to clarify how these mutant proteins interact with Ras and other effectors to deregulate myeloid growth. (Blood. 2004;103:2325-2331)

Introduction

RAS *NF1*

PTPN11

P

PTPN11,

RAS

NF1

P

NF1

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Coded specimens from patients with pediatric acute myelogenous leukemia (AML) were supplied by the National Childhood Cancer Foundation.

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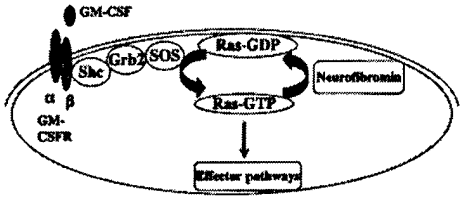

Figure 1. Interaction between Ras and neurofibromin in healthy and JMML cells. GM-CSF binding to its surface receptor leads to dimerization and recruits Janus kinase 2 (JAK2), which creates docking sites for adapter molecules by phosphorylating tyrosine residues on the β chain. The guanine nucleotide dissociation factor SOS induces guanosine diphosphate (GDP) dissociation from Ras at the plasma membrane. Ras is then free to bind to GTP, which interacts with effectors such as Raf-1 to activate kinase signaling cascades. Neurofibromin negatively regulates growth by accelerating hydrolysis of Ras-GTP to inactive Ras-GDP. Oncogenic point *RAS* mutations and loss of *NF1* contribute to aberrant proliferation and survival in JMML by elevating Ras-GTP levels. JMML cells are hypersensitive to GM-CSF in vitro, and studies in stains of mutant mice suggest that GM-CSF plays a central role in the aberrant growth of Nf1-deficient cells in vivo.

activity by altering N-SH2 amino acids that interact with the PTPase domain.[14,15]

SHP-2 participates in signal transduction downstream of growth factor receptors to regulate multiple responses, including proliferation, differentiation, and migration.[19,20] The protein is expressed at high levels in hematopoietic cells and undergoes rapid tyrosine phosphorylation on activation of the c-KIT, interleukin 3 (IL-3), GM-CSF, and erythropoietin receptors.[19,21,22] SHP-2 most often plays a positive role in transducing signals, which is mediated, at least in part, through the Ras/Raf/ERK (extracellular-regulated kinase) cascade in hematopoietic and nonhematopoietic cells.[19,20,23] Loss of *Ptpn11* function has profound effects on the developing hematopoietic system.[24-27] *Ptpn11*-de cient yolk sacs contain markedly reduced numbers of hematopoietic colony-forming cells, and mutant embryonic stem cells do not contribute to hematopoiesis in chimeras.[25] These and other data implicate SHP-2 as a crucial effector of hematopoietic cell fates that modulates signaling from activated growth factor receptors.

Children with NS show a spectrum of hematologic abnormalities, including isolated monocytosis, myeloid disorders with features of chronic myelomonocytic leukemia (CMML) that remit spontaneously and, rarely, JMML.[28-30] Bone marrow cells from NS patients with JMML show characteristic GM-CSF hypersensitivity in methylcellulose cultures.[29] These observations, data implicating hyperactive Ras in the pathogenesis of JMML, and the role of SHP-2 in relaying signals from hematopoietic growth factor receptors to Ras identify *PTPN11* as an excellent candidate gene that might be mutated in cases of JMML without abnormalities in *RAS* or *NF1*. Indeed, a recent study reported somatic *PTPN11* mutations in approximately 35% of JMML samples.[31] We screened a well-characterized panel of JMML specimens for *PTPN11* mutations and con rm that somatic mutations in *PTPN11* are common in this disorder. Genetic evidence implicates these mutations as conferring a growth advantage by deregulating Ras. However, although Ba/F3 cells engineered to express mutant SHP-2 proteins consistently show persistent survival on growth factor withdrawal, biochemical analysis did not reveal hyperphosphorylation of the Ras effectors ERK and Akt. In addition, given the known association of oncogenic RAS mutations in other disorders of myelopoiesis, we screened a number of other patients with myeloid disorders for mutations in *PTPN11*.

Patients, materials, and methods

Leukemia samples

Archived bone marrow or peripheral blood specimens from patients with hematologic malignancies were collected by our laboratory or accrued by the Hematopoietic Tissue Cell Bank of the University of CaliforniaPSan Francisco (UCSF) Comprehensive Cancer Center. Fifty-one patients with JMML; 60 patients with other disorders of myelopoiesis, including acute myelogenous leukemia (AML), chronic myelomonocytic leukemia (CMML), chronic myelogenous leukemia (CML), and myelodysplasia (MDS); and 20 healthy control subjects were screened for mutations in *PTPN11*. An additional 95 DNA samples were analyzed from a national pediatric AML trial conducted by the Children s Cancer Group between 1996 and 2002. Approval for these studies was obtained from the UCSF Committee on Human Research.

Mutation detection

Denaturing high-performance liquid chromatography (DHPLC) facilitates accurate, high-throughput screening of ampli ed genomic DNA for point mutations with a sensitivity of approximately 95%.[32] Polymerase chain reaction (PCR) using previously published primers for exons 1 to 6 and 8 to 15 were performed according to previously published methods.[15] Exon 7 was ampli ed using forward primer 5′GAAGTAATGCTGATCCAGGC3′, reverse primer 5′AAGAGCACACGACCCTGAGG3′, and Accuprime Taq (Invitrogen, Carlsbad, CA). DNA (50-100 ng) was used for each 50 μL reaction. PCR products were visualized on agarose gels prior to DHPLC analysis. Concentrations of primers (Integrated DNA Technologies, Coralville, IA), dNTPs (Roche, Indianapolis, IN), Mg^{++} (Applied Biosystems, Foster City, CA), and Amplitaq Gold (Applied Biosystems), as well as PCR conditions, were optimized. DHPLC was conducted on a Helix HPLC (Varian, Palo Alto, CA) using a DNA Sep column (Transgenomics, Omaha, NE) and analyzed according to previously published methods.[14,15] Abnormal spectrographs were enzyme puri ed using 1 U alkaline shrimp phosphatase (Roche) and 1 U exonuclease I (USB, Cleveland, OH), incubated for 1 hour at 37 C, and heat inactivated for 15 minutes at 95 C. Puri ed products were subsequently sequenced by way of a Prism 3700 Sequencer (ABI, Foster City, CA). The procedures for analyzing *RAS* and *NF1* for mutations have been described.[5,10,12]

SHP-2 expression constructs

Oligonucleotide primers containing attB sites for use with Gateway cloning technology (Life Technologies, Rockville, MD) as well as murine *Ptpn11* gene-speci c sequences were used to amplify cDNA sequences from the ATG start codon to nucleotide 1862. Kozak sequences were incorporated into the primer sequence to allow ef cient translation. Then, 30 cycles of ampli cation using Elongase polymerase (Invitrogen) were used to generate a PCR product for use in the BP reaction to generate an entry clone. Next, using the LR enzyme mix from Gateway technology, *Ptpn11* was cloned into a mouse stem cell virus (MSCV)Pbased retroviral vector[33] containing a puromycin resistance cassette that we modi ed for use with the Gateway system. The QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to generate point mutations into the *Ptpn11* expression constructs, which were con rmed by sequencing.

Analysis of Ba/F3 cells

MSCV-*puro* plasmids engineered to express wild-type SHP-2, the D61Y, or the E76K mutant proteins were cotransfected with plasmids encoding retroviral gag-pol and env proteins into Phoenix cells (a generous gift of Gary Nolan, Stanford University) using Lipofectamine2000 (Invitrogen). Supernatants from transfected cells were used to transduce Ba/F3 cells. Transduced cells were selected for growth in 2.5 μg/mL puromycin for 4 days, and expression of wild-type and mutant SHP-2 was con rmed by Western blot. Ba/F3 parental cells, cells transduced with a control MSCV-*puro* vector, and lines expressing wild-type or mutant SHP-2

proteins were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1 ng/mL recombinant murine IL-3 (Peprotech, Rocky Hill, NJ). After puromycin selection, transduced cells were washed twice in RPMI/10% FBS and grown in IL-3Pfree media. A total of 1×10^6 cells were seeded into 10-cm dishes, and viable cells were counted by trypan blue exclusion for 2 to 3 weeks. To assess signal transduction, Ba/F3 cells were deprived of IL-3 for 6 hours and then stimulated with 10 ng/mL IL-3 for 10 minutes. Cell lysis and immunoblotting for ERK and Akt were performed as previously described.[9] A monoclonal antibody from Transduction Laboratories, Becton Dickinson Biosciences (catalog no. 610621; La Jolla, CA) was used to assess SHP-2 expression.

Results

Missense PTPN11 mutations in JMML specimens

On the basis of case reports of JMML in patients with NS, we first investigated 51 JMML specimens for *PTPN11* mutations, including 2 from children with a clinical diagnosis of NS. These studies uncovered missense mutations in 16 of 49 JMML samples from patients without NS, which are summarized in Table 1. *PTPN11* mutations were also identified in both patients with NS. Representative DHPLC and sequencing data are shown in Figure 2A-B. Fifteen of 16 *PTPN11* mutations detected in sporadic cases of JMML occurred in exon 3, which encodes a segment of the N-SH2 domain (Table 1). The only exception was an exon 4 mutation that we identified in a specimen from a 2-month-old infant. This mutation has also been reported in NS.[15] Although the nature of this *PTPN11* mutation and the early age of diagnosis are suggestive of NS, detailed clinical information was not available in this case. Both of the JMML specimens from children known to have NS demonstrated different *PTPN11* mutations from the patients without NS, including one substitution in exon 13 (Table 1).

We noticed absence of the normal *PTPN11* allele on the DHPLC tracing of a JMML specimen with a mutation at nucleotide 215. This C>T mutation ablates a *Bgl*I cleavage site in the normal sequence and creates a new *Mae*III site. Digestion of PCR products amplified from exon 3 confirmed that the normal allele was absent in this case (Figure 2C). To address whether loss of the normal allele exists in other cases of JMML with *PTPN11* mutations, we performed PCR amplification followed by allele-specific cleavage of 15 additional cases. All of these leukemias retained the normal *PTPN11* allele (data not shown).

Distribution of *PTPN11*, *RAS*, and *NF1* mutations in JMML

If SHP-2 functions in a growth control signaling pathway that includes the GM-CSF receptor, Ras, and neurofibromin in myeloid cells, *PTPN11* mutations might be restricted to leukemia samples without *RAS* or *NF1* mutations. This hypothesis is based on the idea that mutating any component would deregulate the entire cascade and that another mutation would confer little, in any, additional selective advantage. Indeed, previous studies that included most of the JMML specimens investigated in this report revealed *RAS* and *NF1* mutations in mutually exclusive subsets.[5,10,12,13] Similarly, *BRAF* mutations are largely restricted to melanomas without *RAS* mutations.[34] The 49 JMML specimens from patients without NS were divided into 3 groups: (1) samples from patients with a clinical diagnosis of NF1 or an *NF1* mutation, (2) samples with *RAS* (*KRAS* or *NRAS* mutations), or (3) all other JMML samples. When these groups were compared, there was a statistically significant difference in the frequency of *PTPN11* mutations in group 3 versus groups 1 and 2 (Table 2). These data provide genetic evidence that mutant SHP-2 proteins contribute to leukemogenesis through a Ras-dependent mechanism. Two specimens that were assigned to the NF1 group showed *PTPN11* mutations. In one patient with clinical evidence of NF1, extensive molecular analysis did not disclose either loss of the normal *NF1* allele or a truncating

Table 1. *PTPN11* mutations detected in JMML

Nucleotide	Substitution	Amino acid	No. cases
178	G>C	Gly60Arg	1
181	G>T	Asp61Tyr	1
	G>A	Asp61Asn	1
205	G>A	Glu69Lys	1
214	G>A	Ala72Thr	2
215	C>T	Ala72Val	2
218	C>T	Thr73Ile	1 (JMML/NS)
226	G>A	Glu76Lys	3
	G>C	Glu76Gln	1
227	A>G	Glu76Gly	3
417	G>C	Glu139Asp	1
1517	A>C	Gly506Pro	1 (JMML/NS)

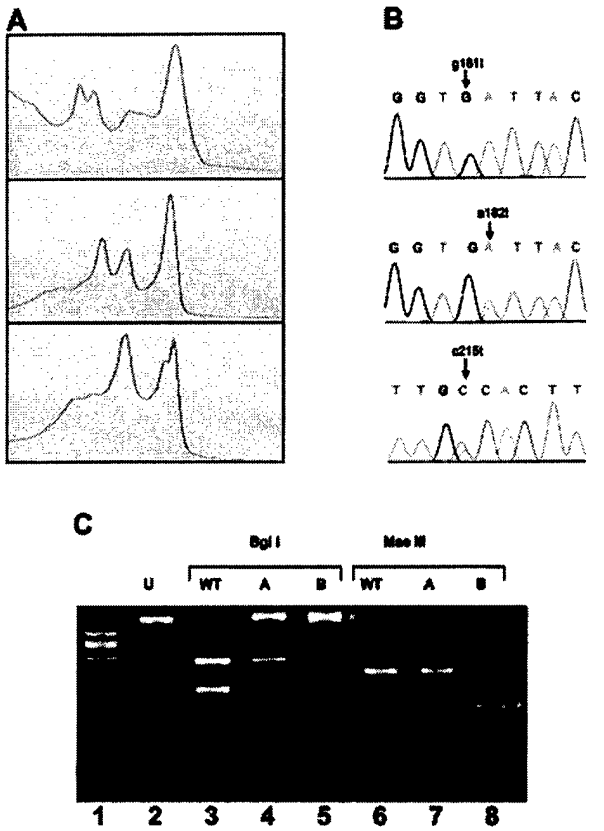

Figure 2. *PTPN11* mutations in JMML specimens. (A) DNA samples from 3 patients with JMML were amplified by using exon 3–specific primers, and the products were analyzed on DHPLC. (B) *PTPN11* mutations corresponding to the abnormal DHPLC spectrographs shown in panel A. Abnormal nucleotides are marked with arrows. (Top) Mutation at nucleotide 181 (G>T); (middle) mutation at nucleotide 182 (A>T); (bottom) mutation at nucleotide 215 (C>T). (C) Absence of the normal *PTPN11* allele in a JMML sample; (lane 1) 1-kb ladder, (lane 2) uncut (u) amplified DNA from healthy bone marrow, (lanes 3-5) DNA amplified from a healthy specimen (WT, lane 3), a patient with a heterozygous mutation (A, lane 4), and the patient with the homozygous mutation (B, lane 5). Note the presence of an abnormal (uncut) band (white asterisk) in both patients, with absence of the normal digested bands in patient B. (Lanes 6-8) The same 3 specimens were cut with *Mae*III, which cleaves at a novel site within the mutant but not the normal allele (lane 6). (Lanes 7-8) An abnormal band (white asterisk) is visible in both patients A and B, and loss of the upper normal band is seen in patient B (lane 8).

Table 2. Incidence of somatic *PTPN11* mutations in subsets of patients with JMML (n = 49)

Patient group	No. cases	No. with mutations	P*
JMML only	20	14	NA
RAS mutation	7	0	< .001
NF1 or *NF1* mutation	22	2	< .001

*Refers to difference between JMML only and other groups (by chi-square test). NA indicates not applicable.

mutation in the coding region in the leukemic clone. Because some patients with NS show clinical features of NF1,[35,36] it is possible that this child was misdiagnosed. The other sample was from a 3-year-old patient without clinical evidence of NF1 who was included in a series of JMML cases studied for *NF1* mutations.[13] Molecular analysis of the bone marrow demonstrated a nonsense mutation (G4614A) in exon 27a of *NF1*, but both *NF1* alleles were retained. It is formally possible that this mutant allele retains some GAP activity, or that this represents an instance in which leukemic outgrowth in an NF1 patient resulted from a somatic *PTPN11* mutation rather than loss of the normal *NF1* allele.

PTPN11 mutations in other hematopoietic malignancies

The prevalence of *PTPN11* mutations in JMML suggested that somatic mutations might also exist in other myeloid malignancies. To address this question, we analyzed specimens from children and adults with CMML, CML, AML, MDS, and therapy-related MDS/AML. *PTPN11* mutations were detected in 7 of these samples (11%), including 1 of 4 from patients with CMML, 0 of 11 with CML, 3 of 28 with AML, 2 of 7 with MDS, and in 1 of 10 with therapy-related MDS/AML (t-MDS/AML). Each mutation was in exon 3 (Table 3), and many of the same mutations were also found in patients with JMML. On the basis of the results of these studies, we performed a focused analysis of exons 3, 4, 5, 8, and 13 in a cohort of 95 pediatric AML specimens and detected 2 mutations in exon 3. The absence of *PTPN11* mutations in CML is consistent with genetic and biochemical data, implicating Ras as a downstream target of Bcr-Abl in myeloid leukemogenesis.[37,38] Five of the 9 samples with *PTPN11* mutations also had chromosome 7 abnormalities (monosomy 7). This nding is of interest, because monosomy 7 has previously been reported in myeloid malignancies with *RAS* or *NF1* mutations, including JMML.[39-43]

Overall, 25 of the 27 *PTPN11* mutations identi ed in JMML and other myeloid malignancies change amino acids within the N-SH2 domain (exon 3) with codons 61, 72, and 76 affected in 4, 6, and 9 cases, respectively (Tables 1,3). By contrast, although exon 3 is also commonly involved in NS, the overall spectrum is much broader with mutations also seen in exons 2, 4, 7, 8, and 13.[14,15] Moreover, only 2 of the leukemia-associated amino acid substitutions identi ed within exon 3 have been reported in NS.[14,15] The JMML specimens analyzed by Tartaglia et al[31] displayed a similar pattern of *PTPN11* mutations. On the basis of the SHP-2 crystal structure, each *PTPN11* mutation is predicted to disrupt the inhibitory interaction of the N-SH2 domain with the PTPase domain (Figure 3A-B).

Table 3. *PTPN11* mutations detected in myeloid malignancies

Nucleotide	Substitution	Amino acid	Diagnosis
179	G>C	Gly60Ala	AML
181	G>T	Asp61Tyr	AML
182	A>T	Asp61Val	MDS
188	A>G	Tyr63Cys	CMML
211	T>C	Phe71Lys	t-MDS
215	C>T	Ala72Val	AML(1) MDS (1)
226	G>A	Glu76Lys	AML
227	A>G	Glu76Gly	AML

Functional and biochemical studies in Ba/F3 cells

Ba/F3 cells have been used to investigate SHP-2 activation in hematopoietic cells[44-46] and to interrogate the functional consequences of leukemia-associated mutant proteins.[47-51] We, therefore, expressed the mutant D61Y and E76K SHP-2 proteins identi ed in samples from patients with JMML in this pro-B cell line, which is dependent on IL-3 for survival and proliferation. Ba/F3 cells were infected with retroviral vectors engineered to coexpress wild-type or mutant SHP-2 with a puromycin resistance gene, then cultured with IL-3 and puromycin. This infection/selection procedure was performed on 3 independent occasions to ensure that the biologic effects of expressing mutant SHP-2 proteins were reproducible. After 4 days, the transduced Ba/F3 cells were transferred to medium without IL-3 to assess the effects of expressing wild-type or mutant SHP-2 on survival and proliferation. At this time, cells were also collected to measure SHP-2 expression and to interrogate Ras effector cascades. Wild-type and mutant SHP-2 proteins were expressed at similar levels that were higher than in parental Ba/F3 cells (Figure 4A, bottom panel). We investigated ERK and Akt phosphorylation in cells that were deprived of serum and IL-3 for 6 hours, then stimulated with IL-3. Surprisingly, we did not observe increased levels of phosphorylated ERK or Akt in resting or IL-3Pstimulated Ba/F3 cells that expressed either mutant SHP-2 protein (Figure 4A). Signal transduction experiments were performed under a variety of experimental conditions that included varying the concentration of IL-3 and the time course with similar results (data not shown). We also assessed the effects of wild-type and mutant SHP-2 proteins on the survival and growth of transduced Ba/F3 cells after IL-3 withdrawal. The cells were cultured in

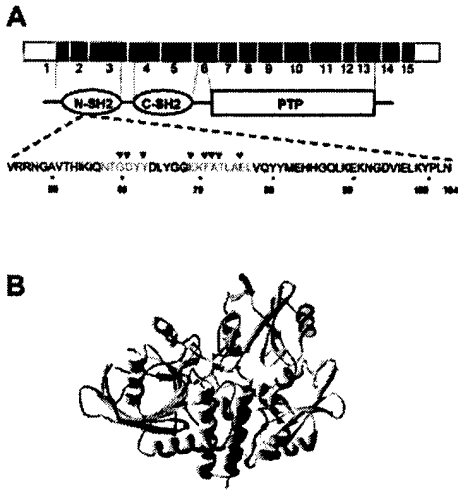

Figure 3. Sites of exon 3 mutations and predicted effects on SHP-2 structure. (A) Schematic of the *PTPN11* gene with functional domains. The amino acid sequence of the N-SH2 domain is highlighted below. The interaction sites between the N-SH2 and PTP domains are indicated in red. The sites of the exon 3 mutations reported here are indicated by the arrowheads. (B) The catalytic cysteine, Cys459, is shown (green dots), as are 2 of the residues mutated in leukemia samples, D61 and E76 (red dots). These residues make critical contacts with the catalytic domain, and the residues' mutation is predicted to disrupt the inhibition of the catalytic domain by the amino-terminal SH2 domain. The N-terminal SH2 domain is shown in blue, the C-terminal SH2 domain in yellow, and the catalytic domain in pink. The figure was generated using Swiss-PdbViewer.

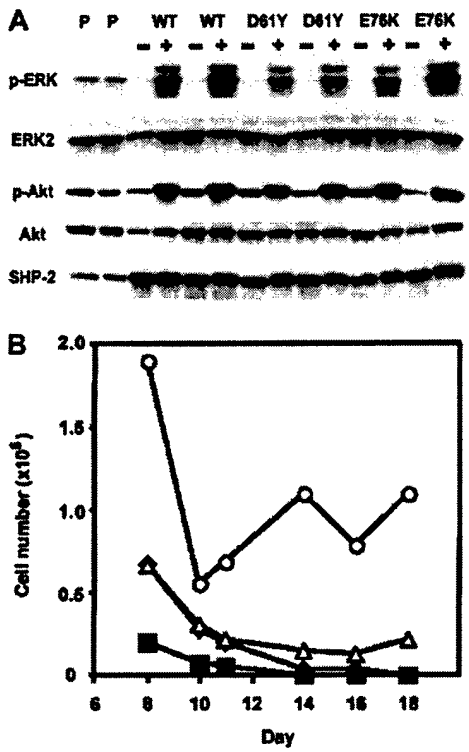

Figure 4. Effects of expressing wild-type and mutant SHP-2 proteins on ERK and Akt activation and survival in Ba/F3 cells. (A) Duplicate aliquots of Ba/F3 cells transduced with retroviruses encoding various SHP-2 constructs (wild-type [WT], D61Y, or E76) were collected after 4 days of growth and selection in medium containing IL-3 and puromycin. Parental Ba/F3 cells are labeled P. The cells were starved for 5 hours and lysed without stimulation (–) or after exposure to 10 ng/mL IL-3 for 10 minutes (+). The bottom panel shows SHP-2 expression, which was equivalent in cells transduced with the WT, D61Y, or E76K vectors and elevated above the levels in parental Ba/F3 cells or in cells infected with the empty vector (not shown). Ba/F3 cells expressing all of the SHP-2 constructs showed low levels of phosphorylated ERK (p-ERK) and Akt (p-Akt) after starvation, with robust and equivalent activation in response to IL-3. Parental and transduced Ba/F3 expressed total levels of ERK2 and Akt. (B) Ba/F3 cell counts after IL-3 withdrawal. Ba/F3 cells infected with the MSCV-*puro* vector (■), and cells expressing either WT SHP-2 (♦), the D61Y SHP-2 mutant protein (△), or the E76K SHP-2 mutant protein (○) were plated in triplicate at 1×10^6 cells/plate in the absence of IL-3. Cells were counted starting on day 8.

triplicate at 1×10^6 cells per plate and were counted every other day beginning on day 8. Under these conditions, expression of the E76K mutant consistently enhanced the survival of Ba/F3 cells (Figure 4B). We also observed subtle, but reproducible, effects of the D61Y SHP-2 protein (Figure 4B). Importantly, Ba/F3 cells transduced with mutant SHP-2 proteins did not expand during the 2- to 3-week culture period, but sustained higher numbers of viable cells. However, Ba/F3 cells that expressed either mutant SHP-2 protein frequently demonstrated growth factorPindependent proliferation after prolonged time in culture. This was never observed in cells that had been transduced with either empty vector or with the wild-type SHP-2 virus.

Discussion

We nd that missense mutations in *PTPN11* are common in JMML and exist in other myeloid malignancies. On the basis of a number of considerations, these amino acid substitutions are almost certain to represent pathologic mutations. First, we did not detect any of these leukemia-associated mutations in 22 healthy bone marrow specimens, and they were not identi ed in more than 100 control subjects screened by Tartaglia et al.[14,15] Second, as would be expected if *PTPN11* mutations result in a gain of function, we have not identi ed deletions, insertions, or substitutions leading to premature termination of protein translation. Third, the data shown in Table 2 argue strongly that these alterations are not random but are functionally equivalent to oncogenic mutations in *RAS* or inactivation of *NF1*. Fourth, on the basis of the crystal structure of SHP-2, each of these mutations is predicted to disrupt the inhibitory interaction of the N-SH2 domain with the PTPase domain (Figure 3A-B). Indeed, in an elegant series of experiments in the *Xenopus* animal cap assay, O Reilly et al[52] generated alanine substitutions corresponding to D61 and E76. These investigators showed that mutant SHP-2 proteins exhibited elevated PTPase activity and conferred a gain-of-function elongation phenotype which is known to involve activation of Ras/ERK signaling downstream of the broblast growth factor (FGF) receptor.[52] Similarly, our data in the Ba/F3 cell line demonstrate that these mutations have phenotypic consequences in hematopoietic cells. Finally, Tartaglia et al[31] independently found a similar incidence and spectrum of somatic *PTPN11* mutations in a different series of JMML samples.

Although many of the somatic *PTPN11* mutations identi ed in leukemia specimens alter the same codons as in NS, the spectrum is distinct with respect to the pattern of amino acid substitutions and speci city for exon 3. Furthermore, the 2 mutations that we detected in JMML specimens from children with NS are uncommon, with only one being previously reported. The distribution of *PTPN11* mutations found in JMML suggests that these alleles might be deleterious in embryonic life. Consistent with this idea, the D61Y and E76K mutant proteins show higher phosphatase activities than the most common substitution found in children with NS (N308D).[31]

Expressing the D61Y and E76K mutations enhanced the survival of transduced Ba/F3 cells that were deprived of IL-3. Interestingly, the E76K mutation, which shows higher PTPase activity,[31] is more potent in this assay. Although mutant SHP-2 proteins did not acutely induce IL-3Pindependent proliferation, there was a lower rate of attrition than in control cells expressing either empty vector or wild-type SHP-2. This is reminiscent of the effects of the E2A-HLF fusion protein in Ba/F3 cells.[47] Despite genetic evidence that the *PTPN11* mutations found in JMML deregulate growth through a Ras-dependent mechanism, we did not detect aberrant activation of ERK or Akt in transduced Ba/F3 cells. Substitutions at the D61 and E76 positions of SHP-2 perturb Ras signaling in other systems.[31,52] The discrepancies between these studies and our data in Ba/F3 cells might be due to differences in the expression levels of mutant proteins and/or the cellular context. It is interesting that Tartaglia et al[31] reported relatively modest levels of ERK activation in COS-7 cells with basal and serum-stimulated kinase levels that were equivalent to wild-type but prolonged activation in cells expressing mutant SHP-2 proteins. Furthermore, although those researchers observed increased proliferation in COS-7 cells, our data in hematopoietic cells support the idea that the predominant effect of mutant SHP-2 proteins is to reduce the requirement for growth factors in cell survival. This model is consistent with data from cultured *Nf1*-de cient myeloid cells, which also survive in the absence of exogenous growth factors.[53]

The absence of hyperphosphorylation of ERK and Akt in transduced Ba/F3 cells is not inconsistent with the idea that mutant SHP-2 proteins deregulate Ras signaling in primary myeloid cells.

However, these data raise the possibility that leukemia-associated mutant alleles undermine myeloid growth control through a Ras-independent mechanism. Indeed, although data from *Drosophila, Xenopus,* and mammalian cells place SHP-2 upstream of Ras in a variety of cell types, other observations support a more complex role that includes functions either parallel to or downstream of Ras.[19,52,54,55] Importantly, SHP-2 phosphatase activity is essential for all of its known biologic functions.[17] Src kinases, which are activated by dephosphorylation, are attractive potential direct or indirect targets of SHP-2 that regulate the growth of many cell types. Biochemical analysis in myeloid lineage cells may uncover how mutant SHP-2 proteins perturb growth control through effects on Ras and other signaling molecules.

Our data raise the possibility that *PTPN11* mutations cooperate with other genetic lesions to induce JMML. This idea is consistent with the clinical observation that hematologic abnormalities, including some JMML-like myeloid disorders, may remit spontaneously in children with NS. Along these lines, it is interesting that we detected loss of the normal *PTPN11* allele in one JMML and a second sample showed both heterozygous inactivation of *NF1* and a *PTPN11* mutation. Our studies to date also suggest that *PTPN11* mutations are relatively common in myeloid malignancies with monosomy 7. Expressing leukemia-associated *PTPN11* alleles in primary murine hematopoietic cells will help to elucidate the cellular and phenotypic consequences of these mutations and the requirement for cooperating events to induce myeloid disease in vivo.

The high prevalence of *PTPN11* mutations in JMML is intriguing and may indicate a speci c role for SHP-2 in regulating GM-CSF signaling, perhaps in the context of fetal and neonatal hematopoiesis. Phosphorylation of Tyr-577 on the β common chain of the activated GM-CSF receptor provides a docking site for Shc, which recruits Grb2, Gab2, SHP-2, and the p85 subunit of phosphatidylinositol-3 kinase (PI3K), and induces downstream activation of Akt.[21,22,45] Interestingly, SHP-2 also interacts directly with β common at Tyr612 and at Tyr695. Although the functional importance of these sites is uncertain, Tyr612 can induce Gab-2 phosphorylation independent of Tyr577.[45] The general idea that mutant SHP-2 molecules might deregulate Ras by aberrantly amplifying signals from activated growth factor receptors is consistent with data from *Xenopus*.[52] In this system, mutant SHP-2 proteins showed elevated PTPase activities and promoted elongation in the absence of FGF. Interestingly, ectopic expression of the D61A and E76A mutants was insuf cient to induce mesoderm induction but reduced the requirement for FGF to complete this process.[52] By analogy, the *PTPN11* mutations found in JMML might contribute to leukemogenesis by hyperactivating Ras signaling at physiologic levels of GM-CSF. This idea is consistent with the hypersensitive pattern of CFU-GM colony growth observed when JMML bone marrows or *Nf1* mutant hematopoietic cells are cultured in methylcellulose and with the profound attenuation of the murine JMML-like myeloproliferative disease with *Gmcsf* ablation.[3,11,56,57] Elucidating how mutant SHP-2 proteins interact biochemically with the GM-CSF receptor, with adapter molecules, and with other phosphotyrosyl substrates in primary myeloid cells will extend our knowledge of normal and leukemic signal transduction. The D61A and E76A mutants created by O Reilly et al[52] retain the capacity to bind phosphotyrosyl substrates, and it will be important to con rm that this is also true of leukemia-associated mutant SHP-2 molecules.

In 1994, Sawyers and Denny[37] pointed out that Ras signaling is perturbed in myeloid malignancies by distinct genetic mechanisms such as oncogenic *RAS* point mutations, the *BCR-ABL* translocation, and *NF1* inactivation. Since then, the FLT3 and c-KIT receptors have joined the list of mutant proteins that appear to contribute to myeloid leukemogenesis, at least in part, through hyperactive Ras. SHP-2 represents the rst tyrosine phosphatase that functions as an oncogene in human cancer and genetic data support the hypothesis that these mutations might deregulate myeloid growth through a Ras-dependent mechanism. However, the biochemical data presented here do not exclude the possibility that other pathways affected by mutant SHP-2 may contribute to myeloid neoplasia. Fully characterizing how SHP-2 relays signals from activated growth factor receptors to Ras and other molecules and how aberrant signals contribute to tumorigenesis may uncover novel therapeutic targets.

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