[CANCER RESEARCH 64, 8816–8820, December 15, 2004]

Advances in Brief

Activating Mutations of the Noonan Syndrome-Associated *SHP2/PTPN11* Gene in Human Solid Tumors and Adult Acute Myelogenous Leukemia

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Abstract

The SH2 domain-containing protein-tyrosine phosphatase PTPN11 (Shp2) is required for normal development and is an essential component of signaling pathways initiated by growth factors, cytokines, and extracellular matrix. In many of these pathways, Shp2 acts upstream of Ras. About 50% of patients with Noonan syndrome have germ-line *PTPN11* gain of function mutations. Associations between Noonan syndrome and an increased risk of some malignancies, notably leukemia and neuroblastoma, have been reported, and recent data indicate that somatic *PTPN11* mutations occur in children with sporadic juvenile myelomonocytic leukemia, myelodysplasic syndrome, B-cell acute lymphoblastic leukemia, and acute myelogenous leukemia (AML). Juvenile myelomonocytic leukemia patients without *PTPN11* mutations have either homozygotic *NF-1* deletion or activating *RAS* mutations. Given the role of Shp2 in Ras activation and the frequent mutation of *RAS* in human tumors, these data raise the possibility that *PTPN11* mutations play a broader role in cancer. We asked whether *PTPN11* mutations occur in other malignancies in which activating *RAS* mutations occur at low but significant frequency. Sequencing of *PTPN11* from 13 different human neoplasms including breast, lung, gastric, and neuroblastoma tumors and adult AML and acute lymphoblastic leukemia revealed 11 missense mutations. Five are known mutations predicted to result in an activated form of Shp2, whereas six are new mutations. Biochemical analysis confirmed that several of the new mutations result in increased Shp2 activity. Our data demonstrate that mutations in *PTPN11* occur at low frequency in several human cancers, especially neuroblastoma and AML, and suggest that Shp2 may be a novel target for antineoplastic therapy.

Introduction

Protein-tyrosine phosphatases (PTPs) have key positive (signal-enhancing) or negative (signal-attenuating) roles in a variety of normal signal transduction pathways. Mutations in PTPs and/or altered expression of PTPs can contribute to disease, including cancer, autoimmune disorders, inflammation, and/or developmental defects (1). The nonreceptor PTP Shp2, encoded by the gene *PTPN11*, is a positive (signal-enhancing) signaling component downstream of growth factor, cytokine, and extracellular matrix receptors and plays an important role in regulating cell growth, transformation, differentiation, and migration. Genetic and biochemical analysis have established that Shp2 is required for normal Ras activation in many of these pathways (1).

Dominant mutations in *PTPN11* cause ~50% of cases of the developmental disorder Noonan syndrome (NS). Furthermore, associations between NS and increased risk of malignancy, notably leukemia (2) and possibly neuroblastoma (3), were reported in early studies. Subsequently, somatic *PTPN11* mutations were found in ~35% of juvenile myelomonocytic leukemias (JMMLs), 10% of childhood myelodysplasic syndromes, and at a lower incidence in other childhood hematopoietic disorders, including B-cell precursor acute lymphoblastic leukemia (~7%) and acute myelogenous leukemia (AML) (~4%) (4–6).

Shp2 has two Src homology 2 domains at its NH_2 terminus (N-SH2 and C-SH2, respectively), a catalytic (PTP) domain, and a COOH terminus containing tyrosyl phosphorylation sites. In the basal state, the PTP domain is inhibited by intramolecular interaction with N-SH2. Phosphotyrosyl peptide binding to the N-SH2 domain induces a conformational change that reverses this inhibition and activates Shp2 (1, 7). Most *PTPN11* mutations in NS and leukemia affect N-SH2 or PTP domain residues involved in basal inhibition of Shp2 (4). The location of these mutations, the crystal structure of Shp2, our work on activated mutants of Shp2 (1), molecular dynamic simulations (8), and functional and biochemical analysis[14] (4) suggest that NS/leukemia mutations are "activated mutants."

Nearly all JMML cases without *PTPN11* mutations have either an activating *RAS* mutation or homozygotic inactivation of the neurofibromatosis type-1 (*NF1*) gene, whose protein product, neurofibromin, is a Ras-GTPase activating protein (RasGap). Given the role of Shp2 in Ras/extracellular signal-regulated kinase (ERK) activation, these findings raise the possibility that Shp2 alterations play a role in other human malignancies that have a low frequency of *RAS* mutations but

Received 6/1/04; revised 9/29/04; accepted 10/22/04.

Grant support: Supported by National Institutes of Health grants R01 CA49152 (B. Neel) and CA43460 (B. Vogelstein) and grants from the International Agency for Research on Cancer and European Molecular Biology Organization (M. Bentires-Alj) and partially supported by the Dana-Farber/Harvard Specialized Programs of Research Excellence in breast cancer.

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[14] H. Keilhack and B. Neel, Distinct biochemical properties of disease-associated SHP2-PTPN11 mutants, manuscript in preparation.

demonstrate an activated Ras/ERK pathway (9). Here, we screened such tumors for *PTPN11* mutations.

Materials and Methods

Complementary RNA and Genomic DNA. Generation of primary lung adenocarcinoma cRNAs was described previously (10). Genomic DNA was extracted from previously characterized human lung cancer cell lines using standard techniques. Genomic DNA from human breast cancer specimens was obtained from the Dana-Farber/Harvard Cancer Center Breast Program Tissue Resource. Paraffin-embedded samples of human gastric cancer were obtained from the Department of Pathology at the Brigham and Women's Hospital (Boston, MA), and DNA was purified using the QIAamp DNA extraction kit (Qiagen, Valencia, CA). Neuroblastoma DNAs were obtained from the Children's Oncology Group Neuroblastoma Nucleic Acids Bank (Children's Hospital of Philadelphia). DNA samples from human colon tumors were described previously (11). DNA samples from prostate cancers were obtained from the Department of Medical Oncology at the Dana-Farber Cancer Institute (Boston, MA); AML, acute lymphoblastic leukemia (ALL), and polycythemia vera (PV) DNAs were provided by the Department of Medicine at the Brigham and Women's Hospital, and melanoma DNAs were from the Department of Pediatric Hematology/Oncology at the Dana-Farber Cancer Institute and Children's Hospital (Boston, MA). DNA from astrocytomas and medulloblastomas was from the Department of Cancer Biology at the Dana-Farber Cancer Institute, and glioblastoma cell lines were from the American Type Culture Collection. All studies were approved by the institutional review boards of the Beth Israel-Deaconess Medical Center and other participating institutions.

Reverse Transcription-Polymerase Chain Reaction, Genomic DNA Polymerase Chain Reaction, and DNA Sequencing. For sequencing cRNAs, reverse transcription-polymerase chain reaction (RT-PCR) was performed using Superscript One-Step RT-PCR and the Platinum Taq kit (Life Technologies, Inc., Gaithersburg, MD), as described previously (12). DNA amplification used M13-tagged primers against three segments of SHP2: segment 1 (first 631 bp), GTAAAACGACGGCCAGTCCTGAGCAAGGAGCGGGT (forward primer) and CAGGAAACAGCTATGACCAGGATTCTTCTTATAATGT (reverse primer); segment 2 (nucleotide 631-1277); GTAAAACGACGGCCAGTGAACTGAAATACGACGTTG (forward primer) and CAGGAAACAGCTATGACCAGTTTAAGTTCTCTTAGCG (reverse primer); and segment 3 (nucleotide 1278–1928), GTAAAACGACGGCCAGTAGTATGCTCTAAAAGAATA (forward primer) and CAGGAAACAGCTATGACCACATCTATTTCTGTGCTGAAG (reverse primer).

PTPN11 exons were amplified from genomic DNAs by polymerase chain reaction (PCR) using exon-specific primers and reamplified by nested PCR using M13-flanked primers (Table 1). Reactions were performed in a 25-μL volume containing 5 ng of genomic DNA, 0.1 μL (0.5 unit) of Platinum Taq DNA polymerase (Life Technologies, Inc.), 1 μL of 10 μmol/L stock solution of each primer, 1 μL of 50 mmol/L $MgCl_2$, 0.5 μL of 10 mmol/L deoxynucleotide triphosphate mix, and 2.5 μL of 10× PCR buffer. Cycling parameters were as follows: 8 minutes at 94°C, 34 cycles of amplification consisting of 45 seconds at 94°C, 30 seconds at 60°C (exons 2, 3, 5, 10, 11, 13, 14, and 15) or 30 seconds at 57°C (exons 4, 6, 7, 8, 9, and 12) and 45 seconds at 72°C, followed by a final extension step of 72°C for 10 minutes. Amplified DNAs were sequenced by Agencourt Bioscience Corp. (Beverly, MA).

Protein-Tyrosine Phosphatase Assays. PTP activity assays were performed using the artificial substrate RCM-lysozyme, essentially as described previously (13).

Results and Discussion

Genomic DNA was obtained from 65 lung cancer cell lines, 9 prostate cancer cell lines, 15 prostate tumors, 100 breast tumors, 40 gastric tumors, 189 colon tumors, 65 AMLs, 11 ALLs, 5 PVs, 10 melanomas, 9 astrocytomas, 9 glioblastomas, 9 medulloblastomas, and 89 neuroblastomas. We also analyzed cRNAs from 118 well-characterized lung tumors (10). The whole *PTPN11* coding region of 118 lung, 24 colon, and 40 gastric carcinomas was sequenced. For the remaining samples, we sequenced either exon 3 alone (165 colon cancers) or exons 2, 3, 4, 5, 7, 8, and 13, which comprise the SH2 and PTP domains (all other neoplasms). These regions were chosen for more intensive sequencing because nearly all reported associated *PTPN11* mutations lie within them (2, 4–6).

Eleven somatic missense mutations of *PTPN11* were found in these samples (Table 2). Five of these are mutations known or predicted to result in an activated form of Shp2. Six of the mutations have not been described previously. We found an additional 87 silent nucleotide changes (Table 3), all of which have been reported previously as single nucleotide polymorphisms (SNPs) in control individuals (2). These changes include 81 intronic SNPs and 6 synonymous changes in exon 3.

Mutations of the *N-* or *K-RAS* genes occur in 15% to 30% of AML patients. In 65 adult AML samples, we found four *PTPN11* mutations: D61Y, a known mutation in childhood leukemia, and three new

Table 1 *Exon-specific and M13 flanked nested PCR primers used in amplification of PTPN11 genomic DNA*

	Forward	Reverse
Exon 2 PCR	CTGATCATATCCCCAGACAC	GGCTCAGATAAGGCCTTCA
Exon 2 nested PCR	GTAAAACGACGGCCAGTACTGAATCCCAGGTCTCTACCAAG	CAGGAAACAGCTATGACCCAGCAAGCTATCCAAGCATGGT
Exon 3 PCR	TCCTTGGGTTTCTTTCAACACT	GCTATTAGTCTCTCTTTAGTAATTC
Exon 3 nested PCR	GTAAAACGACGGCCAGTCGACGTGGAAGATGAGATCTGA	CAGGAAACAGCTATGACCCAGTCACAAGCCTTTGGAGTCAG
Exon 4 PCR	GATTGATCAATCCCTTGGAG	GTCACCAGACCCAACGT
Exon 4 nested PCR	GTAAAACGACGGCCAGTAGGAGAGCTGACTGTATACAGTAG	CAGGAAACAGCTATGACCCATCTGTAGGTGATAGAGCAAGA
Exon 5 PCR	AGTTGTCTCTATATACTAGCTA	GGGTGACAGAGTAAGAC
Exon 5 nested PCR	GTAAAACGACGGCCAGTCTGCAGTGAACATGAGAGTGCTTG	CAGGAAACAGCTATGACCGTTGAAGCTGCAATGGGTACATG
Exon 6 PCR	TGCATTAACACCGTTTTCTGTAATA	TCAAGTCTCTCAGGTCCAATT
Exon 6 nested PCR	GTAAAACGACGGCCAGTTTTCTGTAATATTTTCTTTATTTTACA	CAGGAAACAGCTATGACCCCAATTCCAAACACAAGAG
Exon 7 PCR	CAGATGAACATTCTTGTAGCT	GATGTGCTAACAAGAGCAC
Exon 7 nested PCR	GTAAAACGACGGCCAGTGAACATTTCCTAGGATGAATTCC	CAGGAAACAGCTATGACCGGTACAGAGGTGCTAGGAATCA
Exon 8 PCR	GCATTTTGAGACATCAGGCA	GCTTTGAATTGTTGCACTTGG
Exon 8 nested PCR	GTAAAACGACGGCCAGTGACATCAGGCAGTGTTCACGTTAC	CAGGAAACAGCTATGACCCCTTAAAGTTACTTTCAGGACATG
Exon 9 PCR	ACATCAATGCAAATATCATCATG	AGCTTAATTCATTTATTCAATTCCT
Exon 9 nested PCR	GTAAAACGACGGCCAGTGTAAGCTTTGCTTTTCACAGTG	CAGGAAACAGCTATGACCCTAAACATGGCCAATCTGACAT
Exon 10 PCR	TTCCATGTTGGTGGTTATTAAGCA	TGCATGTATGCAAACATGAGTAAC
Exon 10 nested PCR	GTAAAACGACGGCCAGTGCAAGACTTGAACATTTGTTTGTTGC	CAGGAAACAGCTATGACCGACCCTGAATTCCTACACACCATC
Exon 11 PCR	AGTTGCCTCCAGGATTGCC	CAAAATGTCCCTCAATGCAGTTG
Exon 11 nested PCR	GTAAAACGACGGCCAGTCAAAAGGAGACGAGTTCTGGGAAC	CAGGAAACAGCTATGACCGCAGTTGCTCTATGCCTCAAACAG
Exon 12 PCR	TAGACAATTTTATAAATAGCTCCAAAG	TCAACCTCTCTTCCCCAGA
Exon 12 nested PCR	GTAAAACGACGGCCAGTGCTCCAAAGAGTAGACATTGTTTC	CAGGAAACAGCTATGACCGACTGTTTTCGTGAGCACTTTC
Exon 13 PCR	CTGGGATCATTGGTATTGGT	GTCTATCAGAGCCTGTCC
Exon 13 nested PCR	GTAAAACGACGGCCAGTCAACACTGTAGCCATTGCAACA	CAGGAAACAGCTATGACCCGTATCCAAGAGGCCTAGCAAG
Exon 14 PCR	TCCCCTTAAAATAACCATTGTC	ACACTAACAGTAGGGCAAC
Exon 14 nested PCR	GTAAAACGACGGCCAGTACCATTGTCCCTCACATGTGC	CAGGAAACAGCTATGACCCAGTGAAAGGCATGTGCTACAAAC
Exon 15 PCR	CTATCTCTTCTCTCCCTGG	ACACTTTGCTAAAACATTCCCAAAT
Exon 15 nested PCR	GTAAAACGACGGCCAGTCAGGTCCTAGGCACAGGAACTG	CAGGAAACAGCTATGACCACATTCCCAAATTGCTTGCCT

Table 2 *Mutations of PTPN11* in human cancer

Tumor type	No. of cases	Exon	Mutations
Lung adenocarcinoma (1)	118	3	E76V
Lung cancer cell lines (2)	65	2	**V45L**
		3	**N58S**
Colon cancer (1)	196	3	E76G
AML (4)	65	3	D61Y
		3	**E69V**
		8	**R289G**
		13	**G503V**
Neuroblastoma (3)	89	3	**Y62C**
		3	E69K
		13	T507K
Melanoma (1)	10	4	**R138Q**

NOTE. New mutations are shown in bold. The total number of mutations is indicated in parentheses.

mutations, E69V, R289G, and G503V. Although E69V has not been reported previously, E69Q and E69K have been found in NS and JMML, respectively (4, 14). Asp^{61} and Glu^{69} are located within the N-SH2 domain at the N-SH2/PTP interface. The other two mutations are located in the PTP domain, although only G503V maps to the interface (Fig. 1; Table 2). Both Asp^{61} and Gly^{503} are part of the hydrogen bond network that stabilizes N-SH2 domain binding to the enzyme active site (1). Alterations of the residues in the interface between the "backside" of the N-SH2 and PTP domains would be expected to relieve basal inhibition and yield "activated" mutants. The R289G mutation, however, is not located within the N-SH2/PTP interface.

Four NS patients were reported to develop neuroblastoma, suggesting a possible association with *PTPN11* mutations (3). Indeed, we found three *PTPN11* mutations in 89 primary neuroblastomas surveyed. Two lie within the N-SH2 domain: Y62C, a new mutation (although Y62D is found in NS and JMML), and E69K, a known leukemia-associated mutation. Another new mutation, T507K, lies within the PTP domain (Fig. 1; Table 2). All of the neuroblastoma mutations are located within the N-SH2/PTP interface. The corresponding normal DNA of the first sample (Y62C) harbors the same mutation, indicating that it is a germ-line alteration and that the patient probably has an undiagnosed, poorly penetrant case of NS. The corresponding DNA from normal tissue of the two other patients had the wild-type (WT) *PTPN11* sequence, demonstrating the somatic nature of these mutations.

Neuroblastoma is a malignant childhood tumor of migrating neuroectodermal cells derived from neural crest and destined for the adrenal medulla and the sympathetic nervous system (15). The clinical behavior of neuroblastomas is variable, with occasional spontaneous regression in infants and differentiation into benign ganglioneuroma in some older patients. In most cases, however, neuroblastoma is metastatic at the time of diagnosis and progresses rapidly with a lethal outcome. The mechanisms underlying this diverse behavior remain unclear. *RAS* mutations occur rarely in neuroblastoma, but 4 of 10 human neuroblastoma lines express little or no neurofibromin, and 2 of these lines have *NF1* mutations. It will be important to determine whether *PTPN11* mutation alters clinical outcome.

K-RAS mutations are found in ~30% of pulmonary adenocarcinomas. Furthermore, ~10% of non–small-cell lung carcinoma (NSCLC) patients have *EGFR* mutations (16, 17). Previous work revealed two *B-RAF* mutations in a panel of 127 primary pulmonary adenocarcinomas (12). We sequenced 118 of these samples and found one missense variant (E76V) located within the N-SH2/PTP interface (Fig. 2*B*; Table 2). The corresponding normal tissue lacked this change, proving that this is a *bona fide* tumor-associated mutation (Fig. 2*A*). This tumor had no mutations *in K-RAS*, *B-RAF*, or *EGFR*, suggesting, as discussed below, that these mutations are largely mutually exclusive (10). Analysis of 65 NSCLC lines revealed two additional mutations, both of which were in N-SH2: V45L (HCC1171 cells) and N58S (H661 cells; Table 2; Fig. 1). Val^{45} is located in the N-SH2 domain but maps to the phosphotyrosine peptide-binding pocket rather than the N-SH2/PTP domain interface. Asn^{58} is a key residue in the N-SH2/PTP interface hydrogen bonding network (1, 7), so N58S is probably an activating mutant. Consistent with our findings in neuroblastomas, the H661 cell line has neither *BRAF* nor *RAS* mutations (18). HCC1171 has a G12C *RAS* mutation, however, suggesting that in some cases, *PTPN11* mutations may collaborate with other activating mutations in the Ras/ERK pathway. Although Shp2 is clearly required for Ras/ERK activation, several studies indicate actions downstream or parallel to Ras as well (1).

We also found one known *PTPN11* mutation in a colon tumor: E76G. This residue, which maps to the N-SH2/PTP interface, is a hot spot for JMML mutations. The corresponding normal DNA of this sample lacked this mutation, demonstrating its somatic nature. This particular tumor exhibits "nucleotide instability" with an increased frequency of somatic alterations, but without microsatellite instability. It has WT *RAS*, but a mutated *B-RAF* (R461I).

In melanoma, wherein *B-RAF*, *N-RAS*, or *K-RAS* mutations occur in >60% of cases (19), we found one new mutation (R138Q), located in phosphotyrosyl peptide binding pocket of the C-SH2 domain. This motif is critical for the binding of SH2 domains to tyrosine-phosphorylated residues. The corresponding normal DNA of this sample lacks this mutation, demonstrating that it is not a polymorphism. Because the C-SH2 does not make significant contact with the N-SH2/PTP domain interface, and its role in activation remains controversial, additional experiments are required to address the mechanistic significance of this mutation.

No mutations were found in astrocytoma, glioblastoma, medulloblastoma, ALL, PV, and breast, prostate, and gastric cancers. This could be explained by the low number of samples tested (glioma, PV, and ALL) and/or by the possibility that other oncogenic changes, such as *ERBB2* amplification (breast cancer), can activate the Ras/ERK pathway in these tumors.

Finally, we tested the biochemical effects of several of the new *PTPN11* mutations described herein. PTP assays carried out with the artificial substrate RCM-lysozyme revealed that the N-SH2 mutations V45L (3.5 X), Y62C (2.5 X), and E69K (15.5 X) all were basally activated compared with WT Shp2 (Fig. 2*C*). In contrast, the PTP domain mutation R289G found in an AML patient was not activated and instead showed decreased catalytic activity in this assay. Additional studies will be required to determine whether this mutant is less stable under these conditions, whether it is activated only against

Table 3 *Polymorphisms in PTPN11*

Location	Nucleotide change	Tumor type	No. of cases
Exon 3	255 C→T (H85H)	AML (1)	65
		Breast cancer (2)	100
		Neuroblastoma (3)	89
Intron 4	+12 G→C	Lung cell lines (3)	65
		Breast cancer (5)	100
		AML (1)	65
		PV (1)	5
Intron 7	−21 C→T	Neuroblastoma (20)	89
		Breast cancer (16)	100
		Prostate cancer (6)	24
		Lung cell lines (2)	65
		AML (11)	65
		ALL (2)	11
		Astrocytoma (2)	9
		Glioblastoma (1)	9
		Medulloblastoma (2)	9
	−32 A→C	Neuroblastoma (4)	89
Intron 9	−9 C→A	Gastric cancer (5)	40

NOTE. The total number of nucleotide changes is indicated in parentheses.

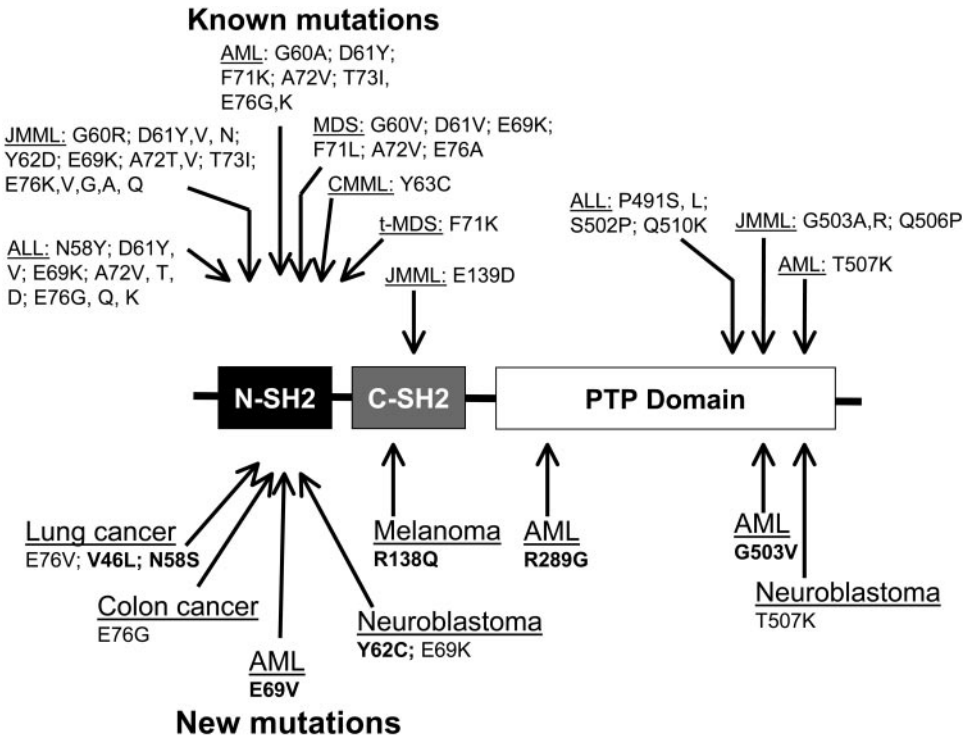

Fig. 1. Distribution of known (*top panel*) and newly discovered (*bottom panel*) *PTPN11* mutations in human cancer. Novel amino acid changes are in *bold*.

some substrates (and not the artificial substrate tested here), and/or whether *PTPN11* mutations can contribute to oncogenesis by mechanisms other than increased basal PTP activity. Furthermore, because we do not have DNA from the normal tissue of this patient, we cannot exclude that R289G is a rare, not previously reported SNP. Notably, V45L, which does appear to be a functionally significant *PTPN11* mutation, is encoded by exon 2, which is often excluded from screens for disease-associated *PTPN11* mutations. This finding, together with data indicating that T42A, a NS-associated mutant, also is enzymatically activated,[14] argues for caution in interpreting negative findings from sequencing only the more commonly affected exons 3 and 13 of *PTPN11*.

RAS mutations are found in many human malignancies (9). Other tumors exhibit ERK activation but have normal *RAS*. Such tumors can have mutations in other Ras/ERK pathway components. For example, >60% of melanomas have *B-RAF* mutations (19), and >60% of colorectal cancers have either *RAS* or *B-RAF* mutations that occur in a mutually exclusive fashion (20). Taken together, the previously described studies of childhood leukemias, the known role of Shp2 as a regulator of the Ras/ERK pathway, and the present findings provide

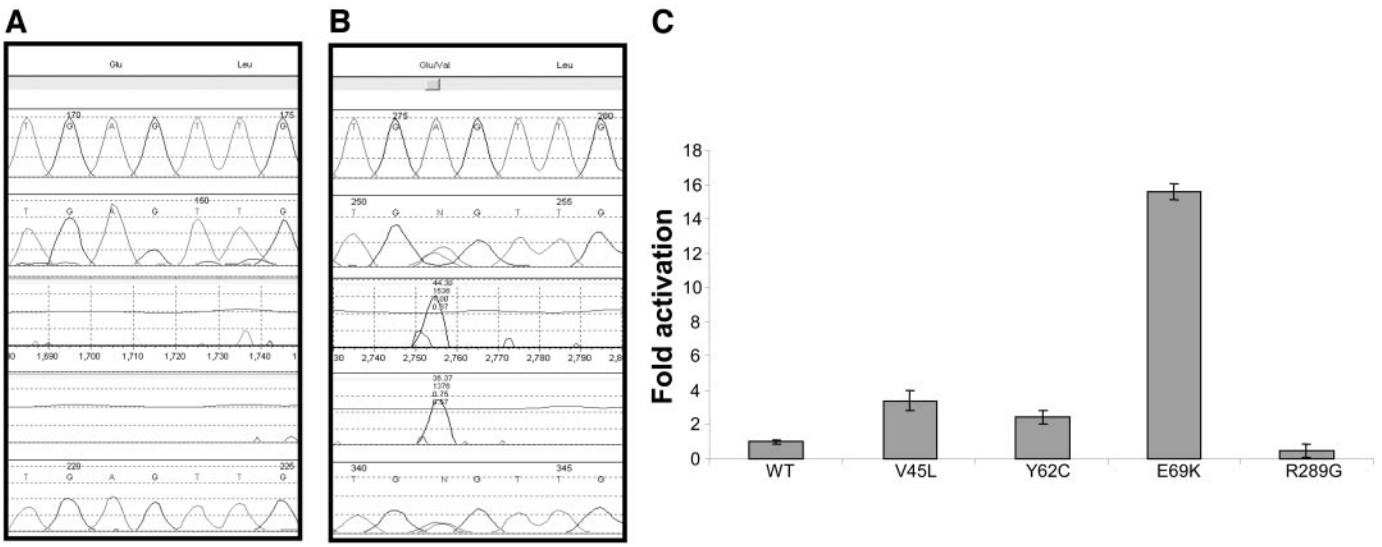

Fig. 2. Selected *PTPN11* mutations in human tumors. DNA sequence of normal tissue (*A*) and in a lung adenocarcinoma (*B*) from the same individual, displayed with Mutation Explorer software (SoftGenetics, State College, PA). The *first box* contains the reference sequence chromatogram, the *second box* contains the forward sequence chromatograms from normal (*A*) or tumor (*B*) tissue, the *third* and *fourth boxes* contain a computed comparison between the sample and the reference displaying a peak at the observed alteration, and the *fifth box* contains the reverse sequence chromatograms. *C*, PTP assays using the artificial substrate RCM-lysozyme and purified recombinant WT Shp2, the lung adenocarcinoma mutant V45L, the AML mutant R289G, and the neuroblastoma mutants E69K and Y62C. Values are mean ± SD of triplicates. All values were normalized to WT Shp2.

evidence that sporadic *PTPN11* mutations contribute to the pathogenesis of other human tumors. Indeed, in preliminary studies, we have found that small interfering RNA-mediated knockdown of Shp2 (WT and N58S mutant) impairs basal and EGF-induced ERK activation in H661 cells (data not shown). Further work is needed to determine the effects of selective elimination of the mutant Shp2 protein in these cells.

Although *PTPN11* mutations are rare, alterations in other signaling molecules have recently been shown to have dramatic pathophysiologic significance. For example, activating *EGFR* mutations are also infrequent but predict clinical response of NSCLC to the EGFR inhibitor geftinib (Iressa) (16, 17). Thus, Shp2 may be a novel target for antineoplastic therapy, particularly in AML and neuroblastoma.

Acknowledgments

We thank Dr. Gordon Chan for help with the figures and the Children's Oncology Group for providing the neuroblastoma DNAs.

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Cancer Res 2004;64:8816-8820.