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# Determination of the catalytic activity of LEOPARD syndrome-associated SHP2 mutants toward parafibromin, a *bona fide* SHP2 substrate involved in Wnt signaling

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ABSTRACT

SHP2, encoded by the *PTPN11* gene, is a protein tyrosine phosphatase that plays a key role in the proliferation of cells via RAS-ERK activation. SHP2 also promotes Wnt signaling by dephosphorylating parafibromin. Germline missense mutations of *PTPN11* are found in more than half of patients with Noonan syndrome (NS) and LEOPARD syndrome (LS), both of which are congenital developmental disorders with multiple common symptoms. However, whereas NS-associated *PTPN11* mutations give rise to gain-of-function SHP2 mutants, LS-associated SHP2 mutants are reportedly loss-of-function mutants. To determine the phosphatase activity of LS-associated SHP2 more appropriately, we performed an *in vitro* phosphatase assay using tyrosine-phosphorylated parafibromin, a biologically relevant substrate of SHP2 and the positive regulator of Wnt signaling that is activated through SHP2-mediated dephosphorylation. We found that LS-associated SHP2 mutants (Y279C, T468M, Q506P, and Q510E) exhibited a substantially reduced phosphatase activity toward parafibromin when compared with wild-type SHP2. Furthermore, each of the LS-associated mutants displayed a differential degree of decrease in phosphatase activity. Deviation of the SHP2 catalytic activity from a certain range, either too strong or too weak, may therefore lead to similar clinical outcomes in NS and LS, possibly through an imbalanced Wnt signal caused by inadequate dephosphorylation of parafibromin.



## 1. Introduction

SHP2 (Src homology 2 domain-containing protein tyrosine phosphatase 2), encoded by the *PTPN11* gene, is a non-receptor-type protein tyrosine phosphatase (PTP) that is evolutionally conserved throughout the Metazoa. SHP2 is ubiquitously expressed throughout body and is intracellularly distributed in both the cytoplasm and the nucleus [1,2]. SHP2 contains two SH2 (N-SH2, C-SH2) domains in its N-terminal region and a catalytic protein tyrosine phosphatase (PTP) domain followed by a C-terminal tail region [3]. In its basal state, SHP2 displays an autoinhibitory configuration that interferes with catalytic reaction to its substrates due to the intramolecular interaction between the N-SH2 domain and PTP domain, therefore maintaining its catalytic activity at a low level. The autoinhibitory interaction is canceled when a tyrosine-phosphorylated protein binds to one or both of the SH2 domains, converting SHP2 to its active form [3].

SHP2 regulates proliferation, differentiation, morphology and motility of cells [3]. In the cytoplasm, SHP2 is activated via SH2 domain-mediated binding with adaptor/scaffold proteins such as Gab proteins, which are tyrosine-phosphorylated by receptor tyrosine kinases. SHP2 is indispensable for full activation of the promitogenic RAS-ERK pathway [4]. Also, SHP2 is translocalized from the cytoplasm to the nucleus through physical complex formation with YAP and TAZ, transcriptional co-activators targeted by the tumor suppressive Hippo signal [5]. In the nucleus, SHP2 dephosphorylates parafibromin, a core component of the RNA polymerase II-associated factor (PAF) complex, making it possible for parafibromin to form a complex with β-catenin. Since the

*Abbreviations:* SHP2, Src homology 2 domain-containing protein tyrosine phosphatase 2; PTP, Protein tyrosine phosphatase; NS, Noonan syndrome; LS, LEOPARD syndrome; PR, Phosphorylation-resistant.

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parafibromin-β-catenin interaction promotes transcription of Wnt target genes, SHP2 potentiates the Wnt signal via tyrosine dephosphorylation of parafibromin in the nucleus [2,6].

Deregulation of SHP2 has been associated with human diseases. Somatic missense mutations in *PTPN11* that constitutively activates SHP2 through abrogation of the autoinhibitory SH2-PTP interaction have been found in hematopoietic malignancies, especially juvenile myelomonocytic leukemia (JMML), as well as non-hematopoietic malignancies such as neuroblastoma and hepatocellular carcinoma, indicating that *PTPN11* is a *bona-fide* oncogene [7]. Germline missense mutations in *PTPN11* account for more than half of the cases of Noonan syndrome (NS) and approximately 90% of the cases of LEOPARD syndrome (LS), both of which are known as leukemia-prone congenital developmental disorders that show multiple overlapping symptoms such as cardiac defects, growth retardation and facial dysmorphism [8,9]. NS-derived *PTPN11* mutations, represented by D61G and N308D, are gain-of–function mutations that give rise to constitutively hyperactivated forms of SHP2, as do mutations found in somatic cancers, as they disrupt the autoinhibitory interaction [10]. In addition to *PTPN11*, mutations in genes encoding components of the RAS-ERK pathway, such as *SOS1*, *RAF1*, *NRAS*, *KRAS* and *BRAF*, have been found in NS patients in a mutually exclusive manner, indicating a central role of dysregulated RAS-ERK signaling in the development of NS [3,7].

LS, which is also recognized as NS with multiple lentigines, is characterized by its major symptoms of multiple Lentigines, Electrocardiographic conduction defects, Ocular hypertelorism, Pulmonary stenosis, Abnormal genitalia, Retardation of growth and sensorineural Deafness [9]. Previous biochemical studies using a standard *in vitro* phosphatase substrate, *p*-nitrophenylphosphate (*p*NPP), showed that major *PTPN11* mutations in LS such as Y279C and T468M, which account for ~65% of total LS cases, gave rise to loss-of-function SHP2 mutants with no catalytic activity [11,12]. Consistently, the LS-derived SHP2 mutant exhibited impaired ability to activate the RAS-ERK pathway [11]. It is therefore mysterious as to how *PTPN11* mutations that affect the SHP2 phosphatase activity in opposite ways, gain-of-function in NS and loss-of-function in LS, contribute to the development of the phenotypically overlapping disorders. To gain insights into the puzzling situation, we investigated enzymatic activity of LS-associated SHP2 mutants toward parafibromin, the *bona fide* SHP2 substrate that regulates nuclear Wnt signaling.

## 2. Materials and methods

### 2.1. Plasmids

The expression vectors for a series of C-terminally Flag-tagged SHP2 were constructed as previously reported [2]. The expression vectors for N-terminally Flag-tagged parafibromin and Bcr-Abl were described previously [2]. pGEX6P2 and pGEX6P1 vectors were used for bacterial expression of SHP2 and parafibromin-6xHis, respectively. Mutagenesis experiments were performed as previously described [2].

### 2.2. Cell culture and transfection

AGS cells and COS-7 cells were cultured as previously described [2]. DNA transfection was performed according to the protocol described previously [2,13].

### 2.3. Antibodies

Anti-SHP2 (C18) and anti-Actin (C11) antibodies were purchased from Santa Cruz. Anti-parafibromin (A300-170A, A300-171A) antibodies were from Bethyl Labs. Anti-phosphotyrosine (4G10) antibody was from Millipore. Anti-flag (M2) antibody was from Sigma–Aldrich. HRP-conjugated IgG were obtained from GE Healthcare.

### 2.4. Immunoprecipitation and substrate trapping experiment

Cell lysates were prepared from COS-7 cells as previously described [2]. Immunoprecipitates made by using an anti-DYKDDDDK (Flag)-tag antibody-conjugated beads (WAKO) were washed with buffer N [250 mM NaCl, 50 mM Tris–HCl (pH 8.0), 5 mM EDTA, 0.5% NP-40, proteinase inhibitors], and then re-suspended in buffer [50 mM Tris–HCl (pH 7.5), 100 mM NaCl] for *in vitro* PTP assay. Substrate trapping immunoprecipitation was performed as described previously [2,13].

### 2.5. Bacterial expression and purification of recombinant proteins

Recombinant proteins were prepared as previously reported [13]. Tyrosine-phosphorylation of recombinant parafibromin was performed by co-expressing v-Src in *Escherichia coli* as described previously [14]. The obtained *E. coli* pellet was re-suspended in Ni-binding buffer [20 mM Tris–HCl (pH 8.0), 100 mM $MgCl_2$, 10 mM imidazole, 0.3 mg/ml benzamidine, 2 mM $Na_3VO_4$], and was then sonicated. After centrifuge, the supernatant was incubated with Ni-NTA agarose beads at 4 °C for 1h. The obtained beads were washed with Ni-wash buffer [20 mM Tris–HCl (pH8.0), 100 mM $MgCl_2$, 20 mM imidazole], and then incubated in Ni-elution buffer [20 mM Tris–HCl (pH8.0), 100 mM $MgCl_2$, 250 mM imidazole]. The eluted fraction was incubated with Glutathione Sepharose 4B beads, and after washing the beads, GST-parafibromin-His was eluted by 10 mM glutathione.

### 2.6. In vitro phosphatase assay

Recombinant GST-pY-parafibromin-His or pY-Flag-parafibromin prepared from COS-7 cells was incubated with recombinant SHP2 proteins (final concentration: 50 μM or 1.5 μM) in buffer containing 2 mM dithiothreitol for 1 h at 37 °C. Phosphatase reaction was terminated by adding SDS buffer. Intensities of the immunoblotted protein bands were quantified by using a LAS-4000 image analyzer (FUJIFILM).

## 3. Results

### 3.1. Expression and analysis of LEOPARD syndrome-associated SHP2 mutants in cells

Most of the missense mutations of the *PTPN11* gene found in LS were characterized by substitutions of the residues composing the catalytic cleft of the PTP domain of SHP2 [7,9]. In this study, we investigated four LS-associated SHP2 mutants, including LS-specific mutants (Y279C, T468M and Q510E) and a mutant found in both LS and NS (Q506P) (Fig. 1A). Mammalian expression vectors for the C-terminally Flag-tagged wild-type SHP2 (SHP2-Flag) and the LS-associated SHP2 mutants (LS-SHP2-Flag) were constructed and then transfected into AGS human gastric epithelial cells (Fig. 1B). Immunofluorescent microscope analysis using an anti-Flag antibody showed that ectopically expressed LS-SHP2 mutants were localized in both the cytoplasm and nucleus, as was the case of wild-type SHP2, in AGS cells (Fig. 1C) [2,5]. We next examined whether LS-SHP2 can undergo enzyme–substrate interaction with parafibromin. It has been reported that the introduction of D425A and C459S substitutions to the catalytic center of the PTP domain enables SHP2 to act as a catalytically inactive, substrate-trapping