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Novel adaptor protein Shf interacts with ALK receptor and negatively regulates its downstream signals in neuroblastoma

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Our neuroblastoma cDNA project previously identified *Src homology 2 domain containing F* (*Shf*) as one of the genes expressed at high levels in favorable neuroblastoma. Shf is an adaptor protein containing four putative tyrosine phosphorylation sites and an SH2 domain. In this study, we found that Shf interacted with anaplastic lymphoma kinase (ALK), an oncogenic receptor tyrosine kinase in neuroblastoma. Real-time PCR analysis showed that *Shf* mRNA is highly expressed in non-metastatic neuroblastomas compared to metastatic tumor samples ($P < 0.030$, $n = 106$). Interestingly, patients showing high *ALK* and low *Shf* mRNA expressions showed poor prognosis, whereas low *ALK* and high *Shf* expressions were related to better prognosis ($P < 0.023$, $n = 38$). Overexpression of ALK and siRNA-mediated knockdown of *Shf* yielded similar results, such as an increase in cellular growth and phosphorylation of ALK, in addition to Erk1/2 and signal transducer and activator of transcription 3 (STAT3) that are downstream signals of the ALK-initiated phospho-transduction pathway. Knockdown of *Shf* also increased the cellular mobility and invasive capability of neuroblastoma cells. These results suggest that Shf interacts with ALK and negatively regulates the ALK-initiated signal transduction pathway in neuroblastoma. We thus propose that Shf inhibits phospho-transduction signals mediated by ALK, which is one of the major key players on neuroblastoma development, resulting in better prognosis of the tumor. (*Cancer Sci* 2013; 104: 563–572)

Neuroblastoma, a solid tumor that accounts for 15% of all pediatric cancer deaths, originates from the sympathoadrenal lineage derived from the neural crest. The clinical behavior of neuroblastoma is markedly heterogeneous.[1] Tumors found in patients under 1 year of age yield favorable prognosis frequently accompanied by spontaneous differentiation and regression, whereas those found in older patients grow aggressively, often resulting in fatal outcomes.[1] Despite the recent treatments and care that have been improved, neuroblastoma harboring the amplified *MYCN* oncogene in an advanced stage is closely correlated to poor outcome.[1,2]

Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase, originally identified as an oncogenic fusion protein nucleophosmin-ALK in anaplastic large cell lymphoma.[3–5] Such unique oncogenic fusion of the *ALK* gene due to chromosomal translocation is responsible for the activation of the ALK signaling pathway in many human cancers including non-small-cell lung cancer.[6–9] Although the expression pattern of ALK in tissues strongly suggests that ALK plays a pivotal role in normal development of the nervous system,[10–12] the molecular mechanism underlying the signal transduction pathway oriented by ALK during neural development and carcinogenesis still remains unclear. Several point mutations that activate the *ALK* gene have been studied in both familial and sporadic cases of neuroblastoma.[13–17] Frequency of point mutations activating ALK in primary neuroblastoma varied between 6% and 11% in these different studies, in which two hot spots of point mutation, *F1174* and *R1275*, were identified.[18] The *F1174* mutation in ALK was linked to a higher degree of autophosphorylation and more potent transforming capacity than the *R1275* mutant.[19] A recent study using transgenic mice indicated that ALK^{F1174L} is sufficient to facilitate neuroblastoma development.[20] In addition, ALK^{F1174L} and MYCN had synergistic effects, as double transgenic mice developed more aggressive neuroblastomas than single transgenic ones of each gene.[21]

Shf (Src homology 2 domain containing F) was originally identified as an adaptor protein homologous to Shb (Src homology 2 domain protein of beta-cells).[22] As the SH2 (Src homology 2) domain[23] at the C-termini is highly conserved among other SH2-containing proteins, they seem to comprise a subfamily of adaptor proteins.[22,24] Although the function of Shf is not fully understood, the SH2 domain is responsible for binding to the platelet-derived growth factor (PDGF)-α receptor at tyrosine 720.[22] Overexpression of Shf significantly decreases the rate of apoptosis induced by PDGF addition, suggesting that Shf is a negative regulator of a receptor-oriented signal pathway.[22]

Our neuroblastoma cDNA project previously identified *Shf* as one of the new genes differentially expressed between favorable and unfavorable subsets of neuroblastoma.[25,26] As we sought to understand how Shf participates in tumorigenesis, the functional relationship between Shf and several receptor tyrosine kinases, such as TrkA and ALK, in neuroblastoma-derived cell lines was examined. Previously, we reported physical interaction between Shf and TrkA.[27] In this work, the regulation of the signal transduction pathway managed by Shf and ALK was investigated in neuroblastoma.

Materials and Methods

Tumor specimens. Neuroblastoma specimens ($n = 106$) used in this study were kindly provided from various institutions and hospitals in Japan to the Chiba Cancer Center Neuroblastoma Tissue Bank (Chiba, Japan). Written informed consent was obtained at each institution or hospital. This study was approved by the Chiba Cancer Center Institutional Review Board. Tumors were classified according to the International Neuroblastoma Staging System (INSS)[28] (25 classified as

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Stage 1; 13 as Stage 2; 31 as Stage 3; 33 as Stage 4; and 4 as Stage 4s). The patients were treated following the protocols proposed by the Japanese Infantile Neuroblastoma Cooperative Study and the Study Group of Japan for Treatment of Advanced Neuroblastoma.[29] Clinical information including age at diagnosis, tumor origin, Shimada histology, prognosis, and survival months of each patient was obtained and used for survival analysis. The median follow-up time for survivors was 52 months (range, 3–208 months). Cytogenetic and molecular biological analysis of all tumors was also carried out by assessing DNA ploidy, *MYCN* amplification, and *TrkA* expression.

Cell culture and transfection. Human neuroblastoma cell lines, SK-N-AS, NLF, SK-N-DZ, and SH-SY5Y were obtained from the CHOP cell line bank (Philadelphia, PA, USA) and maintained in RPMI-1640 (Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated FBS (Invitrogen, Carlsbad, CA, USA), 100 IU/mL penicillin (Invitrogen), and 100 μg/mL streptomycin (Invitrogen), in a humidified atmosphere of 5% CO_2 at 37°C. Human embryonic kidney-derived cell line 293T cells were obtained from Riken BRC Cell Bank (Tsukuba, Japan) and were cultured in DMEM (Nissui) supplemented with 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin. For transient expression, cells were transfected with the indicated expression plasmids using FuGene HD (Roche Applied Science, Mannheim, Germany). For knockdown of endogenous expressions, cells were transfected with 20 nmol/L of indicated siRNAs using Lipofectamine RNAiMax (Invitrogen) and On-Target plus SmartPool (Thermo Fisher Scientific, Waltham, MA, USA). The siRNAs specific to Shf (NM_138356) and ALK (NM_004304) were purchased from Dharmacon (Lafayette, CO, USA).

Cell viability, motility, and invasion assay. Transfected cells were seeded into 96-well plates at 5×10^3 cells/well. Cell viability was measured using a Cell Counting kit-8 (Dojindo Laboratories, Kumamoto, Japan). A BD cell culture insert (#353097) for cell motility assay, and a BD Biocoat Matrigel invasion chamber (#354480) for cell invasion assay were purchased from Becton Dickinson (Franklin Lakes, NJ, USA). Cells were seeded at 2.5×10^4 cells/well and incubated for 23 h in a migratory assay and 27 h in an invasion assay. Migratory cells that penetrated pores on the membrane were fixed with 100% methanol followed by Giemsa staining, and were counted using a conventional light microscope.

Semiquantitative RT-PCR and real-time quantitative RT-PCR. Total RNA was prepared from cultured cells and human tissues, and reverse transcribed using random primers and SuperScript II (Invitrogen), as described previously.[30] Primer sequences for human *Shf* and *GAPDH* mRNA were as follows: Shf-F, 5′-tatgagccagaggaggatgg-3′; Shf-R, 5′-ggcca aggtaggtctttgatg-3′; GAPDH-F, 5′-accacagtccatgccatcac -3′; GAPDH-R, 5′-tccaccaccctgttgctgta-3′. Expression level of *GAPDH* was used as a control. Real-time quantitative RT-PCR was carried out using an ABI PRISM 7500 System (PerkinElmer, Boston, MA, USA). TaqMan probes for Shf (Hs00403125_m1), ALK (Hs00608292_m1), and GAPDH (4310884E) were purchased from Applied Biosystems (Carlsbad, CA, USA). All reactions were carried out in triplicate experiments. The X^2 independence test was used to explore possible associations between expression levels of *Shf* and other factors. Cox regression models were used to explore associations between *Shf*, *ALK*, *TrkA*, ploidy, age, *MYCN*, and survival. $P < 0.05$ was considered significant.

Antibodies. Antibodies were as follows: rabbit anti-Shf antibody raised against SH2 domain and Anti-HA-tag antibody (#561; MBL, Aichi, Japan); human ALK antibodies (#M7195; Dako, Glostrup, Denmark) (#IM3312; Beckman Coulter, Brea, CA, USA); anti-phospho-ALK (Tyr1604) antibody (#3341), anti-Myc-tag antibody (#2276), anti-p44/p42 MAPK, Erk1/2 antibody (#9102), anti-phospho-p44/p42 MAPK (Thr202/Tyr204) antibody (#9101), anti-signal transducer and activator of transcription 3 (STAT3) antibody (#4904), and anti-phospho-STAT3 (Tyr705) antibody (#4113) (Cell Signaling Technology, Danvers, MA, USA); and anti-actin antibody (#sc-8432; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Immunoblotting. Cells were lysed in CHAPS cell extract buffer, separated by 10% SDS-PAGE and transferred onto PVDF membranes (Immobilon-P; Millipore, Billerica, MA, USA). Membranes were incubated with appropriate primary antibodies at room temperature for 2 h, then incubated with HRP-conjugated secondary antibodies at room temperature for 1 h. Immunoreactive bands were visualized using the ECL system (GE Healthcare, Chalfont St Giles, UK). Developed signals were analyzed using a LAS-4000 imager (GE Healthcare).

Immunoprecipitation. Transfected 293T cells lysed in CHAPS cell extract buffer were mixed with indicated antibodies and rotated for 3 h at 4°C. The immune complexes were precipitated with Protein G (GE Healthcare) Sepharose beads for 1 h of incubation at 4°C by rotation. Beads were then washed with Wash buffer (50 mM PIPES, 2 mM EDTA, 150 mM NaCl, 0.1% Triton X-100); immunoprecipitated proteins were eluted from beads using 100 mM glycine (pH 2.5), boiled with SDS sample buffer, and immunoblotted.

Immunofluorescence stain. Transfected 293T cells seeded onto cover slips were fixed with 4% formaldehyde and permeabilized with 0.1% Triton X-100 containing PBS. Cells were then incubated with appropriate antibodies at room temperature for 2 h then incubated with goat anti-rabbit IgG antibody conjugated with Alexa Fluor 488 (Molecular Probes, Invitrogen) and goat anti-mouse IgG antibody conjugated with Alexa Fluor 546 at room temperature for 1 h in the dark. The cells were enclosed with Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA), and observed under a Leica confocal microscope (Wetzlar, Germany).

Results

High *Shf* mRNA expression significantly associated with better prognosis in neuroblastoma. We have reported many candidate genes for novel prognostic factors of neuroblastoma[25,26] in a differential expression study using our cDNA collection prepared from the primary samples of neuroblastoma patients. Among them, *Shf* was identified as one of the possible tumor suppressor genes in neuroblastoma. Shf, a homolog of Shb, has a highly conserved SH2 domain in the C-termini, but lacks a proline-rich region and phosphotyrosine-binding (PTB) domain in the N-termini (Fig. 1a). The expression level of *Shf* was closely correlated with favorable prognosis of neuroblastoma (Fig. 1b). To further confirm the expression profile of *Shf* mRNA, 106 clinical samples were classified into two groups in regard to INSS stages (Fig. 1c). The expression level of *Shf* was higher in a non-metastatic group (INSS 1, 2, and 3) than in metastatic one (INSS 4 and 4s); the classification with favorable (INSS 1, 2, and 4s) and unfavorable groups (INSS 3 and 4) did not yield statistical significance (Fig. S1). A low level of *Shf* expression had significant correlation with poor prognostic factors, such as lower expression of *TrkA* ($P < 0.001$), DNA diploidy ($P < 0.001$), and the patients who contracted the disease after 1 year of age ($P < 0.05$), whereas no significant correlation was observed with the copy number of *MYCN* (Fig. 1d).

Another adaptor protein Shb, a homolog of Shf, interacts with several receptor tyrosine kinases and regulates such receptor-oriented signal transduction pathways. Thus, we

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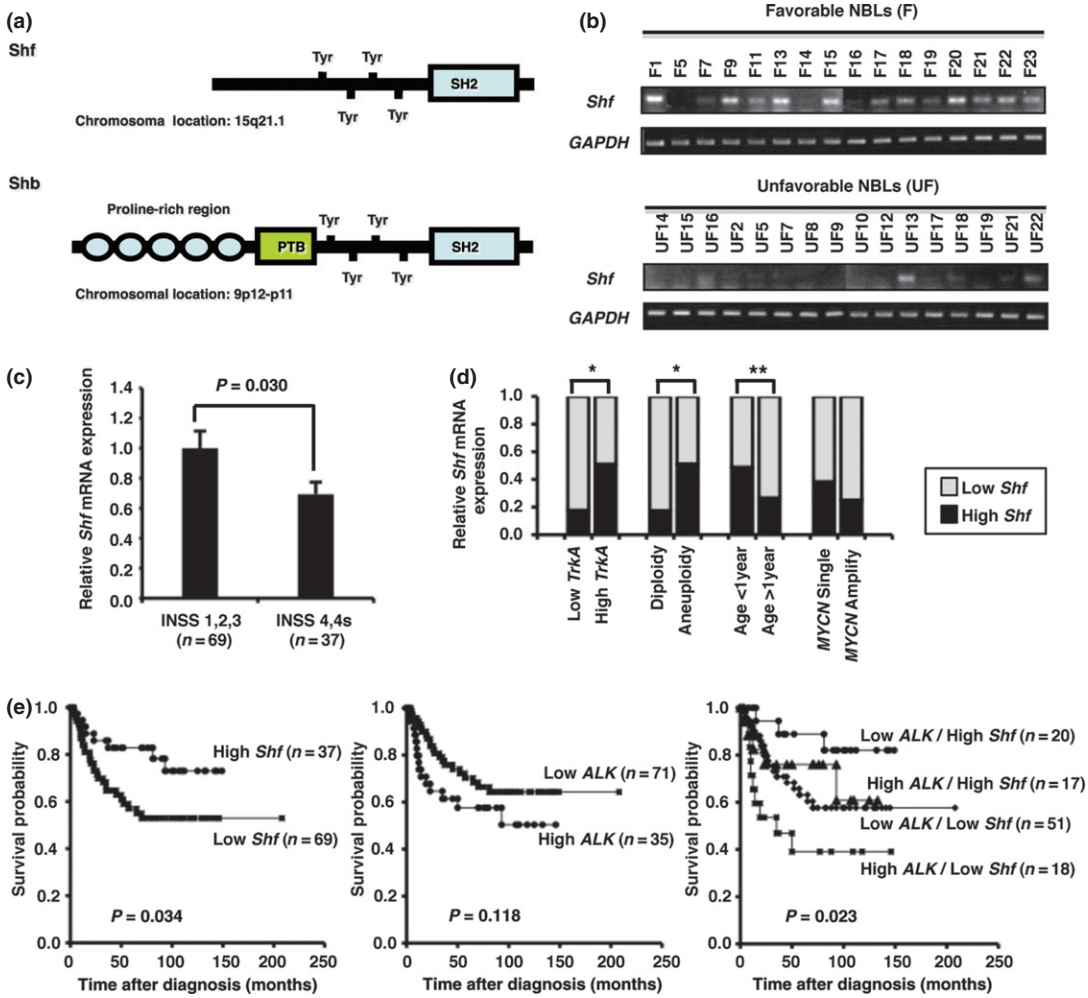

Fig. 1. Expression profiles of *Shf* mRNA in primary neuroblastoma (NBL). (a) Structural differences between Shf and Shb adaptor proteins. PTB, phosphotyrosine-binding domain; SH2, Src homology 2 domain; Tyr, tyrosine. (b) Differential expression of *Shf* in neuroblastomas with favorable (F) and unfavorable (UF) outcomes. Results of 16 representative clinical samples of each group are shown. *GAPDH* was used as a control. Favorable NBLs, stage 1 or 2, with single copy of *MYCN*. Unfavorable NBLs, stage 3 or 4, with *MYCN* amplification. (c) Relative *Shf* expression profiles regarding metastatic status in NBL specimens measured by quantitative real-time PCR. *Shf* mRNA expression was normalized to that of *GAPDH*. Values are shown as means ± SEM. Non-metastatic group, stages 1–3; metastatic group, stages 4 or 4s. (d) Correlation between *Shf* expression and other prognosis factors in NBL. The χ^2-test was used to explore possible associations. $*P < 0.001$; $**P < 0.05$. (e) Kaplan–Meier cumulative survival curves of *Shf* and anaplastic lymphoma kinase (*ALK*) expressions. High and low levels of *Shf* and *ALK* were determined based on mean values.

hypothesized that Shf also participates in the regulation of the signal pathway through its interaction with receptor tyrosine kinases including TrkA and ALK that play critical roles in the nervous system.[10,12,31] Intriguingly, *Shf* was specifically expressed in diencephalon, spinal cord, and dorsal root ganglion in mice.[27] Additionally, we showed that *Shf* was particularly expressed in human brain (Fig. S2a). Therefore, we used statistical analyses to clarify the relationship among these factors and survivability of neuroblastoma patients. The log–rank test indicated that a low level of *Shf* expression is significantly correlated to the number of deaths, as well as other prognostic factors,[32] such as low level of *TrkA* expression, DNA diploidy, age diagnosed after 1 year, and the amplification of *MYCN* copy number, whereas *ALK* expression had no significant correlation (Table 1). Univariate analysis using the Cox regression model yielded similar results (Table 2). Multivariate analysis indicated that *Shf* was not independent compared to other prognostic factors (Table 3), suggesting that *Shf* expression cannot be used as a new prognostic factor in neuroblastoma. Consistent with these statistical analyses, Kaplan–Meier cumulative survival curves indicated that higher expression of *Shf* is significantly correlated with favorable outcome (Fig. 1e). Although it is not statistically significant, higher expression of *ALK* shows some relevance to unfavorable outcome. To further confirm these results, 106 samples were classified into four groups in regard to the expression levels of *Shf* and *ALK* and the survival curves were examined. The patients with lower *Shf* and higher *ALK* were significantly associated with unfavorable outcome, whereas those with higher *Shf* and lower *ALK* yielded markedly favorable results. These results suggest that there is an inverse correlation between expression levels of *Shf* and *ALK* in terms of the clinical prognosis in neuroblastoma.

Physical interaction between Shf and ALK and their colocalization in the juxtamembrane region in 293T cells. As these statistical analyses suggested the functional relationship between Shf and ALK, we asked whether these two proteins have direct interaction *in vivo*. Toward this, we carried out

Table 1. Analysis of relationships between *Shf, ALK*, and *TrkA* expression and other prognostic factors in neuroblastoma patients, using the log–rank test

	No. of patients	No. of deaths	Mean ± SEM	*P*-value
Shf expression				
Low	69	29	0.53 ± 0.07	0.0345*
High	37	8	0.73 ± 0.09	
ALK expression				
Low	71	22	0.64 ± 0.06	0.1178
High	35	15	0.50 ± 0.10	
TrkA expression				
Low	52	26	0.45 ± 0.08	<0.0005*
High	51	9	0.78 ± 0.07	
DNA ploidy				
Aneuploidy	47	4	0.43 ± 0.09	<0.0001*
Diploidy	43	23	0.90 ± 0.05	
Age				
<1 year	42	5	0.88 ± 0.05	<0.0005*
>1 year	64	32	0.43 ± 0.07	
MYCN copy number				
Single	81	18	0.73 ± 0.06	<0.0001*
Amplification	25	19	0.20 ± 0.09	

*$P < 0.05$.

Table 3. Multivariate analysis of *Shf, ALK*, and *TrkA* expression and other prognostic factors in neuroblastoma patients using Cox regression model

Multivariate analysis		*P*-value	HR (95%CI)
A	*Shf* (low *vs* high)	0.253	1.7 (0.7–3.9)
	TrkA (low *vs* high)	0.002*	3.4 (1.5–7.5)
B	*Shf* (low *vs* high)	0.014*	2.7 (1.2–6.1)
	ALK (low *vs* high)	0.031*	2.1 (1.1–4.1)
C	*Shf* (low *vs* high)	0.260	1.9 (0.6–5.7)
	DNA ploidy (diploidy *vs* aneuploidy)	0.001*	6.3 (2.1–19.1)
D	*Shf* (low *vs* high)	0.163	1.8 (0.8–3.9)
	Age (<1 year *vs* >1 year)	0.002*	4.4 (1.7–11.4)
E	*Shf* (low *vs* high)	0.116	1.9 (0.9–4.2)
	MYCN (single *vs* amplification)	<0.001*	5.4 (2.8–10.3)
F	*Shf* (low *vs* high)	0.052	2.2 (1.0–4.8)
	Tumor origin (adrenal gland *vs* others)	0.032*	2.1 (1.1–4.2)
G	*Shf* (low *vs* high)	0.358	1.5 (0.6–3.6)
	Shimada histology (favorable *vs* unfavorable)	<0.001*	8.1 (3.1–21.5)
H	*Shf* (low *vs* high)	0.069	2.1 (0.9–4.6)
	INSS stage (1, 2, 4s *vs* 3, 4)	<0.001*	9.1 (2.8–29.7)

*$P < 0.05$. CI, confidence interval; HR, hazard ratio; INSS, International Neuroblastoma Staging System.

Table 2. Univariate analysis of *Shf, ALK*, and *TrkA* expression and other prognostic factors in neuroblastoma patients using Cox regression model

Univariate analysis		*n*	*P*-value	HR (95%CI)
A	*Shf* (low *vs* high)	69 *vs* 37	0.039*	2.3 (1.0–5.0)
B	*ALK* (low *vs* high)	71 *vs* 35	0.121	1.7 (0.9–3.2)
C	*TrkA* (low *vs* high)	52 *vs* 51	<0.001*	3.9 (1.8–8.4)
D	DNA ploidy (diploidy *vs* aneuploidy)	47 *vs* 43	<0.001*	7.6 (2.6–22.1)
E	Age (<1 year *vs* >1 year)	42 *vs* 64	<0.001*	4.9 (1.9–12.6)
F	*MYCN* (single *vs* amplification)	81 *vs* 25	<0.001*	5.8 (3.0–11.1)

*$P < 0.05$. CI, confidence interval; HR, hazard ratio.

immunoprecipitation using the cell lysate prepared from 293T cells in which exogenous Shf and ALK are overexpressed, and proved reciprocal interaction between ALK and Shf (Fig. 2a). To further confirm this result, we used several point mutants of ALK that were recently reported in neuroblastoma.[13–17] *F1174L* and *R1275Q* are the "hot spot" mutations in the kinase motif located in the intracellular domain of ALK, whereas the *A1099T* mutation is located in the transmembrane domain. Immunoprecipitation indicated that Shf could interact with all of these mutated constructs of ALK, as well as wild-type (Fig. 2b). There are minor differences in the binding capability of Shf to each ALK mutant, possibly suggesting that these point mutations in ALK may affect the affinity to Shf. In addition, immunofluorescence stain indicated that exogenous Shf and ALK were enriched at the cellular membrane (Fig. 2c), suggesting that two proteins colocalized at the juxtamembrane region in 293T. Taken together, we concluded that Shf binds to ALK *in vivo*.

Overexpression of ALK facilitated cellular growth. It has been reported that ALK is an oncogenic receptor tyrosine kinase that transmits survival signals in several cell lines and tissues from different origins.[33] Consistent with previous reports,[34–37] successful overexpression of ALK induced phosphorylation of Erk1/2 and STAT3 even in neuroblastoma cells, such as SK-N-DZ, SK-N-AS, and NLF, clearly suggesting that abundant ALK affects the downstream of signal transduction pathway oriented by ALK (Fig. 3a). Overexpression of ALK also increased the number of cells, indicating that ALK may play an important role during the development of neuroblastoma (Fig. 3b). In contrast, overexpression of Shf affects neither the phosphorylation of ALK (Tyr1604) and downstream factors (Fig. 3c) nor cellular growth (Fig. 3d).

Knockdown of *Shf* promoted Erk1/2 and STAT3 phosphorylation and enhanced cell growth. The results of Kaplan–Meier survival analyses suggested that *Shf* had a biological function opposite to oncogenic *ALK*. Thus, we used a knockdown strategy to investigate the cellular property of Shf in neuroblastoma. The expression of *Shf* mRNA was efficiently inhibited by siRNA transfection in three neuroblastoma cells, SK-N-DZ, SK-N-AS, and NLF (Fig. 4a), that express low levels of wild-type *ALK* (Fig. S2b). Knockdown of *Shf* accelerated phosphorylation of Erk1/2 in SK-N-DZ and SK-N-AS as well as ALK itself at tyrosine 1604. In addition, phosphorylation of STAT3 was observed by *Shf* knockdown in SK-N-DZ and NLF (Fig. 4b). Knockdown of *Shf* enhanced cell growth in these cells, which was statistically significant (Fig. 4c). Next, we used a combination of siRNAs specific to *Shf* and *ALK* in neuroblastoma cell line SH-SY5Y, in which ALK has the *F1174L* mutation (Fig. 4d) and Shf is expressed (Fig. S2b). Knockdown of *Shf* increased the growth rate of SH-SY5Y in the presence of endogenous *ALK* (Fig. 4e). However, under the experimental condition that *ALK* was suppressed by specific siRNA (Fig. 4d, lower panel), *Shf* knockdown did not facilitate cell growth (Fig. 4e). This result indicates that the acceleration of cell growth rate mediated by knockdown of *Shf* depends on *ALK*, suggesting that Shf inhibits growth signals that are downstream of the ALK-initiated signal transduction pathway in neuroblastoma.

Depletion of *Shf* facilitated cell migration and invasion of neuroblastoma cells. Various fusion proteins of ALK exert oncogenic properties (e.g. increasing migration in fibroblast and lymphoid cells)[38,39] and suppression of *Shf* might

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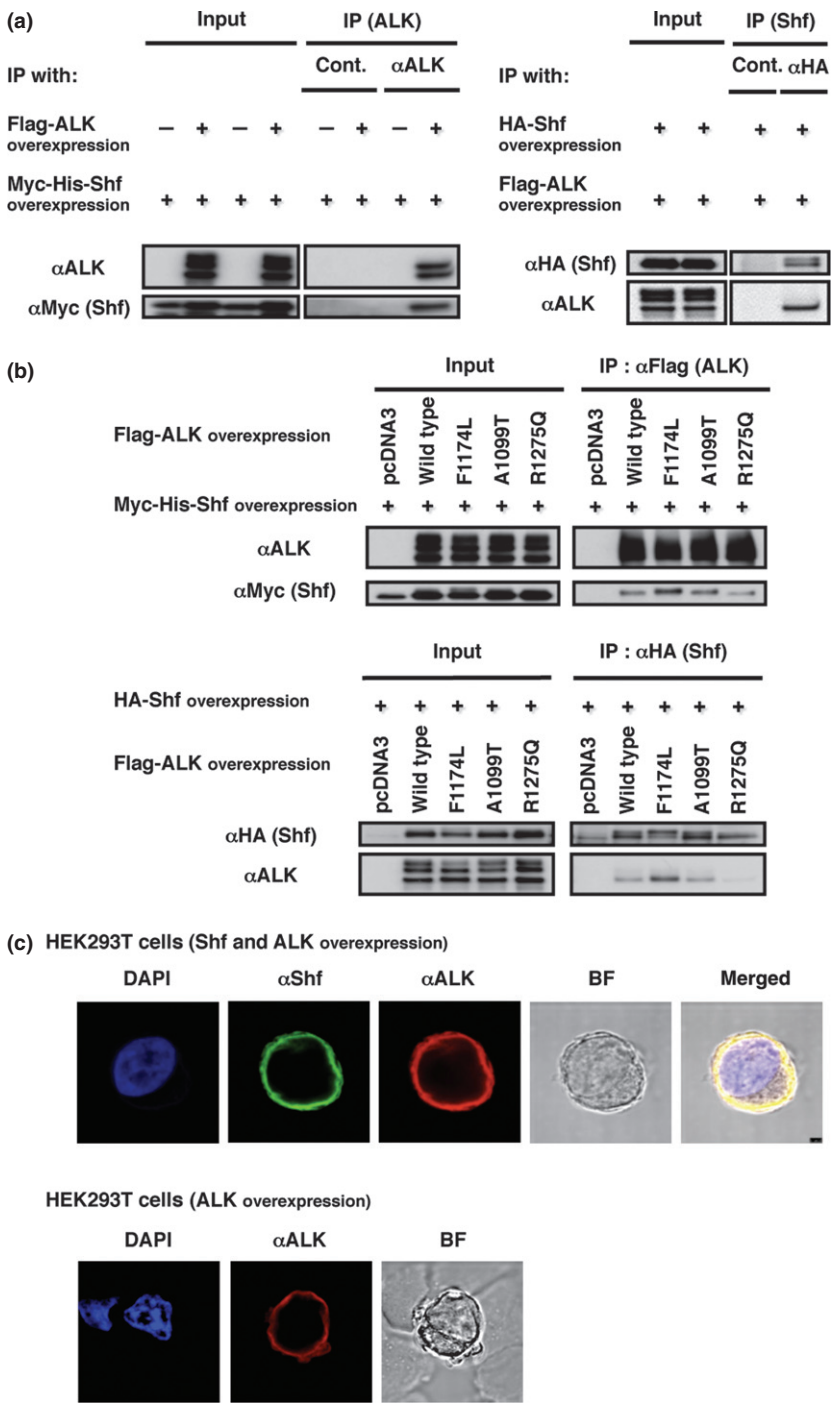

Fig. 2. Physical interaction between adaptor protein Shf and anaplastic lymphoma kinase (ALK). (a) Immunoprecipitation (IP) in 293T cells. Flag-tagged ALK and either HA-tagged or Myc-His-tagged Shf were exogenously overexpressed. Cont., control. (b) Immunoprecipitation assay under the exogenous expression of ALK mutants and Shf in 293T cells. (c) Subcellular colocalization of Shf and ALK in human embryonic kidney (HEK) 293T cells. Myc-His-Shf and Flag-ALK were overexpressed in 293T and indirect immunofluorescence staining was carried out. Upper panels: DAPI (blue), Shf (green), ALK (red), blight field (BF), and merged images. Lower panels: exogenous expression of ALK alone yielded a similar localization pattern at the juxtamembrane region, indicating that the localization of ALK was not affected by Shf overexpression.

positively affect the consequence of ALK activation. To prove this possibility, we examined the ALK-promoted cell motility and invasive ability of neuroblastoma cells under the condition that *Shf* was suppressed. Knockdown of *Shf* greatly increased the number of migrated cells in both NLF and SK-N-DZ cells, compared to the corresponding control (Fig. 5a). As well, *Shf* knockdown in NLF yielded a significant increase in the number of invasive cells. There was a mild tendency of increasing invasion in SK-N-DZ, although it was not statistically significant (Fig. 5b). These results suggest that suppression of *Shf* promotes the motility and invasive capability of neuroblastoma cells, which is consistent with our clinical data that lower expression of *Shf* was observed in metastatic primary neuroblastoma defined by INSS 4 and 4s (Fig. 1c).

Finally, we sought to confirm the biological function of Shf as a negative regulator in ALK-promoted cell mobility. Toward this, overexpression of ALK and siRNA-mediated suppression of *Shf* was carried out simultaneously. The increase of migration mediated by *Shf* knockdown was enhanced more than twofold when ALK was overexpressed (Fig. 5c). While either knockdown of Shf (Fig. 3a) or overexpression of ALK (Fig. 5d) facilitated phosphorylation of ALK, simultaneous treatment of Shf suppression and ALK overexpression further promoted the phosphorylation of ALK itself (Fig. 5d). The combination of Shf suppression and

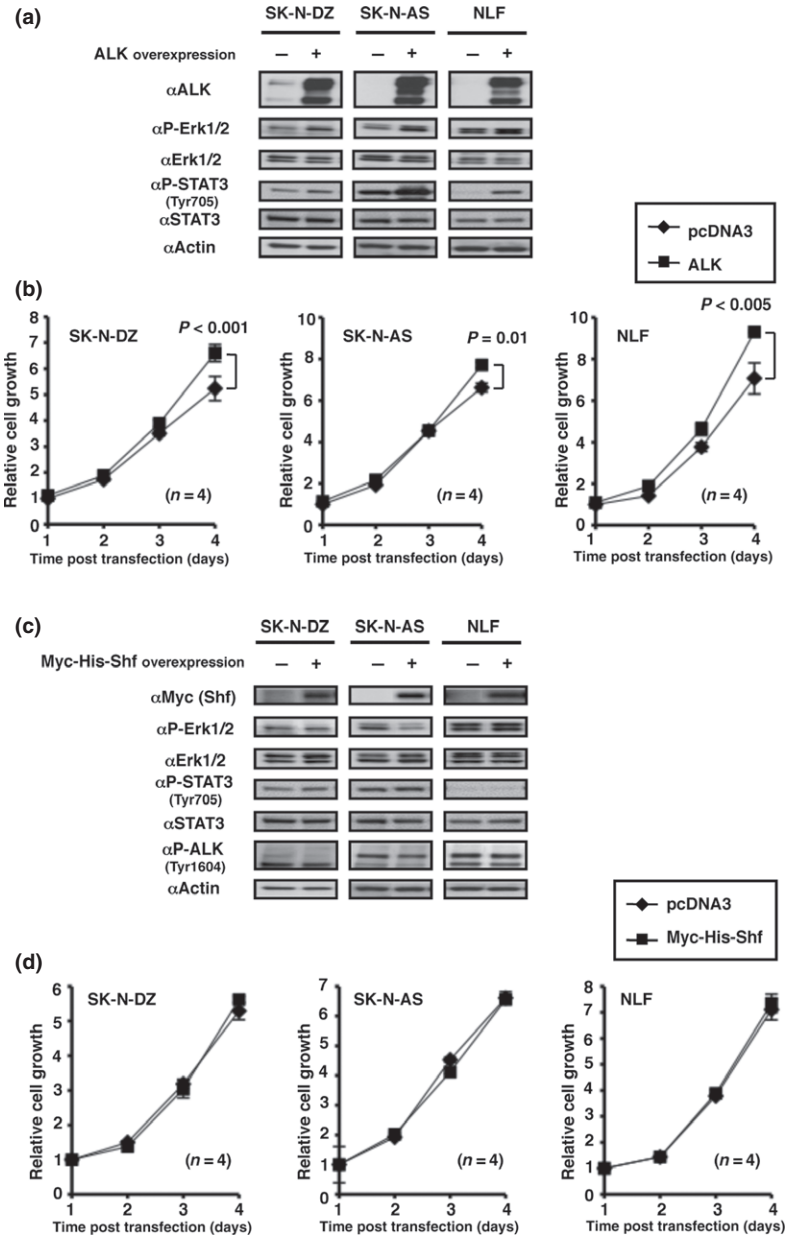

Fig. 3. Overexpression of anaplastic lymphoma kinase (ALK) facilitates cell growth and activates downstream signal pathways. (a) ALK overexpression induced phosphorylation of Erk1/2 and signal transducer and activator of transcription 3 (STAT3) in neuroblastoma cell lines SK-N-AS, SK-N-DZ, and NLF. (b) Cell growth promoted by exogenous expression of ALK. Growth rate was measured by WST assay. Mean values were calculated from quadruplicate experiments. Error bars show standard deviation. Contrarily, overexpression of Shf had least effect on the ALK signaling pathway (c) and cell growth (d).

ALK overexpression in NLF also yielded an increase of phosphorylation status of STAT3 at tyrosine 705, compared to individual treatment (Fig. 5d). These results suggest that Shf inhibits phosphorylation of ALK and STAT3, phospho-transduction signals that are downstream of ALK activation.[34,37,40] Therefore, we concluded that Shf negatively regulates phospho-transduction signals in ALK-oriented pathways, resulting in modulation of cell mobility and invasiveness in neuroblastoma.

Discussion

In this work, we identified that an adaptor protein Shf is a negative regulator of ALK and its downstream signals in neuroblastoma. High levels of *Shf* mRNA expression were observed in neuroblastomas with favorable outcome, whereas low expression was associated with unfavorable tumors. Shf interacts with ALK *in vivo*, suggesting the molecular function of Shf participating in ALK-oriented signal transduction pathways during neural development and tumorigenesis. In the absence of ALK, however, knockdown of *Shf* did not facilitate cell growth; overexpression of ALK stimulated the effect of *Shf* knockdown, suggesting that Shf inhibits the downstream signal initiated by ALK. Therefore, we concluded that the adaptor protein Shf interacts with ALK receptor and modulates oncogenic activity in neuroblastoma.

As an adaptor protein containing the SH2 domain, it can be implied that Shf may play multifunctional roles in a variety of aspects of cellular activity, depending on the interaction with different receptor proteins. Indeed, adaptor proteins bind to receptors at the cell membrane and regulate signal transduction pathways either positively or negatively. For instance, Shf suppresses a signal transduction initiated by PDGFα receptor, resulting in inhibition of apoptosis.[22] In contrast, Shb, another SH2-containing adaptor protein highly homologous to Shf, facilitates the PDGFα-oriented signal, leading to activation of apoptosis.[41] Structural differences between Shf and Shb may explain the molecular mechanism of this contradictory result.

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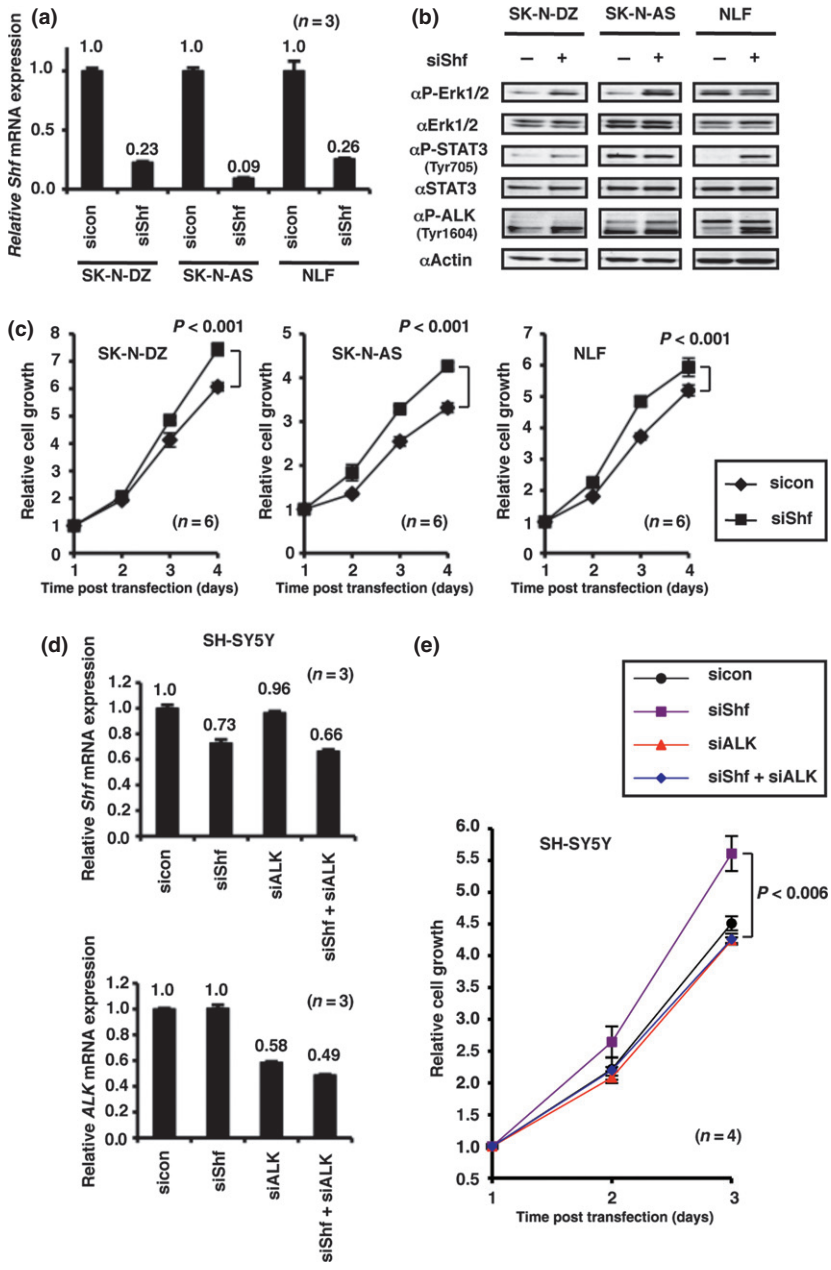

Fig. 4. Knockdown of *Shf* facilitates cell growth as well as activation of the anaplastic lymphoma kinase (ALK) pathway. (a) Knockdown of *Shf* mRNA mediated by specific siRNA was confirmed by real-time PCR. (b) *Shf* knockdown induced phosphorylation of ALK itself, Erk1/2, and signal transducer and activator of transcription 3 (STAT3) in neuroblastoma-derived cell lines. (c) Cell growth was facilitated when *Shf* was knocked down. (d) siRNA-mediated knockdown of *Shf* and *ALK*, confirmed by real-time PCR. The siRNA specific to *Shf* alone or those to *Shf* and *ALK* were used. (e) Growth effect of *Shf* knockdown in the presence or absence of *ALK*. Mean values of quadruplicate experiments are shown. sicon, siRNA control.

Compared to Shb, Shf lacks the PTB domain and proline-rich motifs at the N-termini, whereas the C-terminal region containing the SH2 domain is highly conserved (Fig. 1a). The SH2 domain is responsible for the binding to the receptor; the PTB domain is necessary to activate PDGFα. Therefore, Shf may act as a dominant negative competitor to Shb. In the case of ALK receptor tyrosine kinase, it has been well studied that ShcC, which is also a member of the SH2 adaptor protein family, facilitates the phospho-signal transduction initiated by ALK, inducing survival signals.[36,42] In this work, we showed that Shf negatively regulates the ALK signaling pathway, resulted in inhibition of cell growth and motility. This novel inhibitory mechanism mediated by Shf on the ALK signal pathway may confer the molecular model how adaptor proteins regulate phospho-transduction pathways that manage cell growth and mobility.[43–47]

This work showed that Shf physically binds to ALK and negatively regulates signal transduction downstream of the ALK pathway in neuroblastoma. Knockdown of *Shf* promoted phosphorylation of Erk/STAT accompanied by an increase in cell growth rate. Interestingly, this effect was nullified when ALK was simultaneously knocked down, indicating that existence of ALK is a prerequisite for suppression of ALK-oriented signal transduction mediated by Shf. This result suggested that Shf negatively regulates downstream of the ALK signal pathway. In addition, an increase of cell migration capability by *Shf* knockdown was significantly stimulated when ALK was exogenously overexpressed, further supporting the notion above. It should be noted that overexpression of ALK increased the growth of cells, but overexpression of Shf alone had no such effect (Fig. 3b, d). We speculate that this is due to the titration out of ALK protein by abundant Shf. This may also explain why Shf showed higher affinity with a constitutively active mutant (*F1174L*) of ALK than with wild-type (Fig. 2b). Abundant Shf protein may not be able to affect the mutant form of

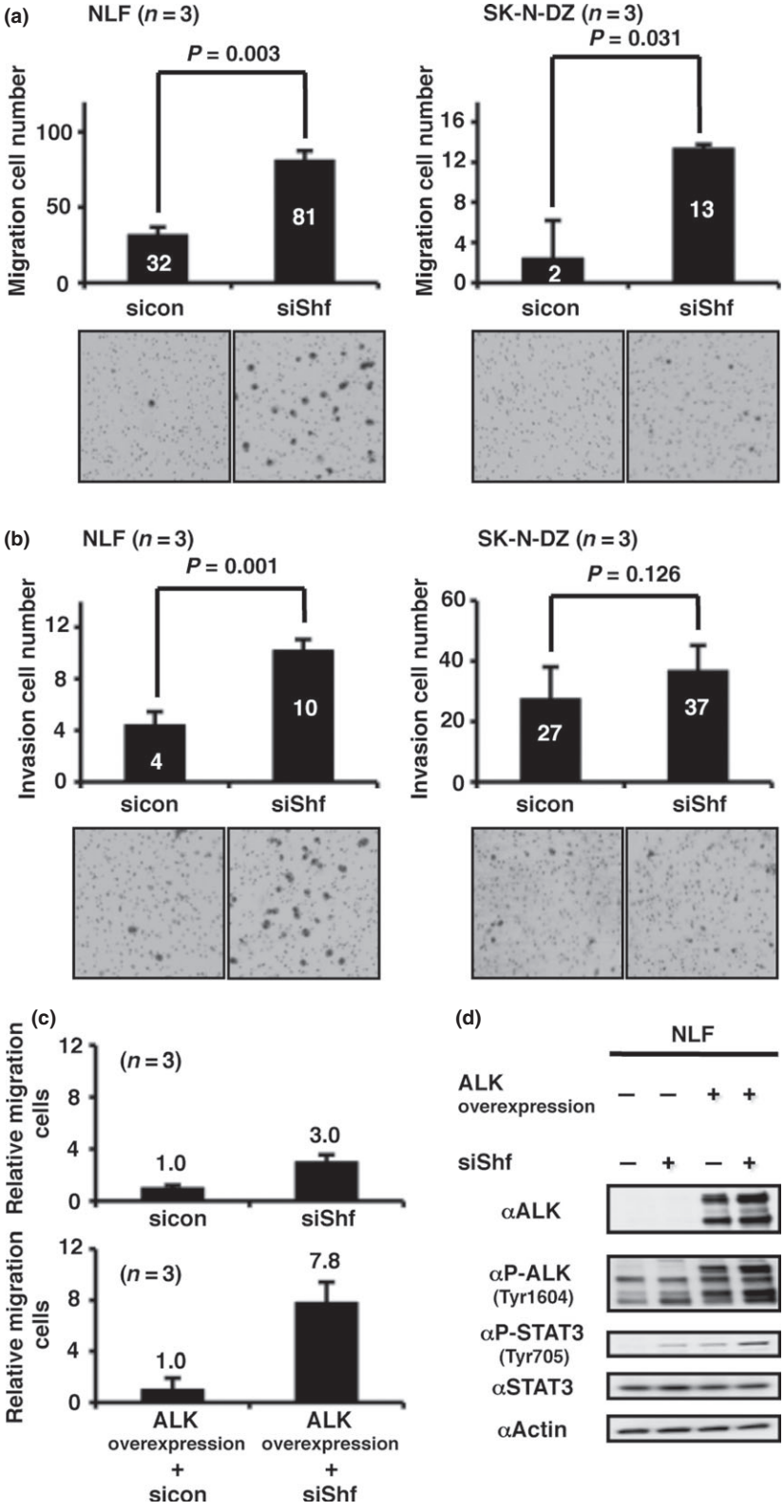

Fig. 5. Knockdown of *Shf* mediated by siRNA increases cellular motility and invasion capability in NLF and SK-N-DZ neuroblastoma cell lines. (a) Cellular migration was stimulated by knockdown of *Shf*. Mean values were calculated from independent triplicate experiments. Error bars indicate standard deviation. Representative blight field images are also shown. (b) Cellular invasion was promoted by *Shf* knockdown in neuroblastoma cell lines. (c) Cellular migration activity was stimulated by *Shf* knockdown. Cell migration assay was carried out in the presence or absence of expression vector of anaplastic lymphoma kinase (*ALK*). (d) *Shf* knockdown facilitated phosphorylation of ALK and signal transducer and activator of transcription 3 (STAT3) under the condition that ALK was overexpressed. sicon, siRNA control.

ALK, while the interaction between these two proteins was facilitated.

The ALK kinase inhibitor crizotinib (PF-02341066) reportedly inhibits proliferation of cells that express *R1275Q*-mutated ALK, whereas cells harboring *F1174L*-mutated ALK were relatively resistant.[48] In contrast, a small molecular weight compound TAE-684, another ALK inhibitor, decreased proliferation of human neuroblastoma cell lines harboring *F1174L*-mutated ALK.[15] Treatment of ALK^{F1174L} transgenic mice with TAE-684 induced complete tumor regression.[20] Therefore, combinations of the addback of Shf and the use of ALK inhibitors may be helpful to develop a potential treatment and cure for neuroblastoma.

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Disclosure Statement

The authors have no conflicts of interest.

References

1 Brodeur GM. Neuroblastoma: biological insights into a clinical enigma. *Nat Rev Cancer* 2003; **3**: 203–16.
2 Maris JM. The biologic basis for neuroblastoma heterogeneity and risk stratification. *Curr Opin Pediatr* 2005; **17**: 7–13.
3 Morris SW, Kirstein MN, Valentine MB *et al.* Fusion of a kinase gene, ALK, to a nucleolar protein gene, NPM, in non-Hodgkin's lymphoma. *Science* 1994; **263**: 1281–4.
4 Shiota M, Nakamura S, Ichinohasama R *et al.* Anaplastic large cell lymphomas expressing the novel chimeric protein p80NPM/ALK: a distinct clinicopathologic entity. *Blood* 1995; **86**: 1954–60.
5 Fujimoto J, Shiota M, Iwahara T *et al.* Characterization of the transforming activity of p80, a hyperphosphorylated protein in a Ki-1 lymphoma cell line with chromosomal translocation t(2;5). *Proc Natl Acad Sci USA* 1996; **93**: 4181–6.
6 Griffin CA, Hawkins AL, Dvorak C, Henkle C, Ellingham T, Perlman EJ. Recurrent involvement of 2p23 in inflammatory myofibroblastic tumors. *Cancer Res* 1999; **59**: 2776–80.
7 Jazii FR, Najafi Z, Malekzadeh R *et al.* Identification of squamous cell carcinoma associated proteins by proteomics and loss of beta tropomyosin expression in esophageal cancer. *World J Gastroenterol* 2006; **12**: 7104–12.
8 Soda M, Choi YL, Enomoto M *et al.* Identification of the transforming EML4–ALK fusion gene in non-small-cell lung cancer. *Nature* 2007; **448**: 561–6.
9 Rikova K, Guo A, Zeng Q *et al.* Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. *Cell* 2007; **131**: 1190–203.
10 Iwahara T, Fujimoto J, Wen D *et al.* Molecular characterization of ALK, a receptor tyrosine kinase expressed specifically in the nervous system. *Oncogene* 1997; **14**: 439–49.
11 Morris SW, Naeve C, Mathew P *et al.* ALK, the chromosome 2 gene locus altered by the t(2;5) in non-Hodgkin's lymphoma, encodes a novel neural receptor tyrosine kinase that is highly related to leukocyte tyrosine kinase (LTK). *Oncogene* 1997; **14**: 2175–88.
12 Vernersson E, Khoo NK, Henriksson ML, Roos G, Palmer RH, Hallberg B. Characterization of the expression of the ALK receptor tyrosine kinase in mice. *Gene Expr Patterns* 2006; **6**: 448–61.
13 Caren H, Abel F, Kogner P, Martinsson T. High incidence of DNA mutations and gene amplifications of the ALK gene in advanced sporadic neuroblastoma tumours. *Biochem J* 2008; **416**: 153–9.
14 Chen Y, Takita J, Choi YL *et al.* Oncogenic mutations of ALK kinase in neuroblastoma. *Nature* 2008; **455**: 971–4.
15 George RE, Sanda T, Hanna M *et al.* Activating mutations in ALK provide a therapeutic target in neuroblastoma. *Nature* 2008; **455**: 975–8.
16 Janoueix-Lerosey I, Lequin D, Brugieres L *et al.* Somatic and germline activating mutations of the ALK kinase receptor in neuroblastoma. *Nature* 2008; **455**: 967–70.
17 Mosse YP, Laudenslager M, Longo L *et al.* Identification of ALK as a major familial neuroblastoma predisposition gene. *Nature* 2008; **455**: 930–5.
18 Janoueix-Lerosey I, Schleiermacher G, Delattre O. Molecular pathogenesis of peripheral neuroblastic tumors. *Oncogene* 2010; **29**: 1566–79.
19 De Brouwer S, De Preter K, Kumps C *et al.* Meta-analysis of neuroblastomas reveals a skewed ALK mutation spectrum in tumors with MYCN amplification. *Clin Cancer Res* 2010; **16**: 4353–62.
20 Heukamp LC, Thor T, Schramm A *et al.* Targeted expression of mutated ALK induces neuroblastoma in transgenic mice. *Sci Transl Med* 2012; **4**: 141ra91.
21 Berry T, Luther W, Bhatnagar N *et al.* The ALK(F1174L) mutation potentiates the oncogenic activity of MYCN in neuroblastoma. *Cancer Cell* 2012; **22**: 117–30.
22 Lindholm CK, Frantz JD, Shoelson SE, Welsh M. Shf, a Shb-like adapter protein, is involved in PDGF-alpha-receptor regulation of apoptosis. *Biochem Biophys Res Commun* 2000; **278**: 537–43.
23 Welsh M, Mares J, Karlsson T, Lavergne C, Breant B, Claesson-Welsh L. Shb is a ubiquitously expressed Src homology 2 protein. *Oncogene* 1994; **9**: 19–27.
24 Oda T, Kujovich J, Reis M, Newman B, Druker BJ. Identification and characterization of two novel SH2 domain-containing proteins from a yeast two hybrid screen with the ABL tyrosine kinase. *Oncogene* 1997; **15**: 1255–62.
25 Ohira M, Morohashi A, Inuzuka H *et al.* Expression profiling and characterization of 4200 genes cloned from primary neuroblastomas: identification of 305 genes differentially expressed between favorable and unfavorable subsets. *Oncogene* 2003; **22**: 5525–36.
26 Ohira M, Morohashi A, Nakamura Y *et al.* Neuroblastoma oligo-capping cDNA project: toward the understanding of the genesis and biology of neuroblastoma. *Cancer Lett* 2003; **2**: 63–8.
27 Furuya T, Kamijo T, Ozaki T, Kusafuka T, Nakagawara A. Functional implication of the Shf in Neuroblastoma. *Nichidai Igaku Zasshi* 2006; **65**: 367–75.
28 Brodeur GM, Pritchard J, Berthold F *et al.* Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment. *J Clin Oncol* 1993; **11**: 1466–77.
29 Kaneko M, Nishihira H, Mugishima H *et al.* Stratification of treatment of stage 4 neuroblastoma patients based on N-myc amplification status. Study group of Japan for treatment of Advanced Neuroblastoma, Tokyo, Japan. *Med Pediatr Oncol* 1998; **31**: 1–7.
30 Machida T, Fujita T, Ooo ML *et al.* Increased expression of proapoptotic BMCC1, a novel gene with the BNIP2 and Cdc42GAP homology (BCH) domain, is associated with favorable prognosis in human neuroblastomas. *Oncogene* 2006; **25**: 1931–42.
31 Nakagawara A. Trk receptor tyrosine kinases: a bridge between cancer and neural development. *Cancer Lett* 2001; **169**: 107–14.
32 Nakagawara A, Arima-Nakagawara M, Scavarda NJ, Azar CG, Cantor AB, Brodeur GM. Association between high levels of expression of the TRK gene and favorable outcome in human neuroblastoma. *N Engl J Med* 1993; **328**: 847–54.
33 Chiarle R, Voena C, Ambrogio C, Piva R, Inghirami G. The anaplastic lymphoma kinase in the pathogenesis of cancer. *Nat Rev Cancer* 2008; **8**: 11–23.
34 Zamo A, Chiarle R, Piva R *et al.* Anaplastic lymphoma kinase (ALK) activates Stat3 and protects hematopoietic cells from cell death. *Oncogene* 2002; **21**: 1038–47.
35 Wan W, Albom MS, Lu L *et al.* Anaplastic lymphoma kinase activity is essential for the proliferation and survival of anaplastic large-cell lymphoma cells. *Blood* 2006; **107**: 1617–23.
36 Osajima-Hakomori Y, Miyake I, Ohira M, Nakagawara A, Nakagawa A, Sakai R. Biological role of anaplastic lymphoma kinase in neuroblastoma. *Am J Pathol* 2005; **167**: 213–22.
37 Palmer RH, Vernersson E, Grabbe C, Hallberg B. Anaplastic lymphoma kinase: signalling in development and disease. *Biochem J* 2009; **420**: 345–61.
38 Armstrong F, Duplantier MM, Trempat P *et al.* Differential effects of X-ALK fusion proteins on proliferation, transformation, and invasion properties of NIH3T3 cells. *Oncogene* 2004; **23**: 6071–82.
39 Dupuis-Coronas S, Lagarrigue F, Ramel D *et al.* The nucleophosmin-anaplastic lymphoma kinase oncogene interacts, activates, and uses the kinase PIKfyve to increase invasiveness. *J Biol Chem* 2011; **286**: 32105–14.
40 Hirano T, Ishihara K, Hibi M. Roles of STAT3 in mediating the cell growth, differentiation and survival signals relayed through the IL-6 family of cytokine receptors. *Oncogene* 2000; **19**: 2548–56.
41 Hooshmand-Rad R, Lu L, Heldin CH, Claesson-Welsh L, Welsh M. Platelet-derived growth factor-mediated signaling through the Shb adaptor protein: effects on cytoskeletal organization. *Exp Cell Res* 2000; **257**: 245–54.
42 Miyake I, Hakomori Y, Shinohara A *et al.* Activation of anaplastic lymphoma kinase is responsible for hyperphosphorylation of ShcC in neuroblastoma cell lines. *Oncogene* 2002; **21**: 5823–34.
43 Van der Geer P, Wiley S, Lai VK *et al.* A conserved amino-terminal Shc domain binds to phosphotyrosine motifs in activated receptors and phosphopeptides. *Curr Biol* 1995; **5**: 404–12.
44 Pelicci G, Dente L, De Giuseppe A *et al.* A family of Shc related proteins with conserved PTB, CH1 and SH2 regions. *Oncogene* 1996; **13**: 633–41.

45 Tamir I, Cambier JC. Antigen receptor signaling: integration of protein tyrosine kinase functions. *Oncogene* 1998; **11**: 1353–64.
46 Downward J. Ras signalling and apoptosis. *Curr Opin Genet Dev* 1998; **8**: 49–54.
47 Miyake I, Hakomori Y, Misu Y *et al.* Domain-specific function of ShcC docking protein in neuroblastoma cells. *Oncogene* 2005; **24**: 3206–15.
48 Bresler SC, Wood AC, Haglund EA *et al.* Differential inhibitor sensitivity of anaplastic lymphoma kinase variants found in neuroblastoma. *Sci Transl Med* 2011; **3**: 108ra14.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Relative Shf expression profiles in favorable/unfavorable samples.

Fig. S2. Tissue and cell line specificities of Shf and anaplastic lymphoma kinase (ALK).

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