5. Vogelstein, B., and Kinzler, K. W. (1992) p53 function and dysfunction. *Cell* **70,** 523–526
6. Velculescu, V. E., and El-Deiry, W. S. (1996) Biological and clinical importance of the p53 tumor suppressor gene. *Clin. Chem.* **42,** 858–868
7. Vogelstein, B., Lane, D., Levine, A. J. (2000) Surfing the p53 network. *Nature* **408,** 307–310
8. Donehower, L. A., Harvey, M., Slagle, B. L., McArthur, M. J., Montgomery, C. A., Jr., Butel, J. S., and Bradley, A. (1992) Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* **356,** 215–221
9. el-Deiry, W. S., Kern, S. E., Pietenpol, J. A. (1992) Definition of a consensus binding site for p53. *Nat. Genet.* **1,** 45–49
10. Sionov, R. V., and Haupt, Y. (1999) The cellular response to p53. The decision between life and death. *Oncogene* **18,** 6145–6157
11. Prives, C., and Hall, P. A. (1999) The p53 pathway. *J. Pathol.* **187,** 112–126
12. Haupt, Y., Maya, R., Kazaz, A., and Oren, M. (1997) Mdm2 promotes the rapid degradation of p53. *Nature* **387,** 296–299
13. Kubbutat, M. H., Jones, S. N., and Vousden, K. H. (1997) Regulation of p53 stability by Mdm2. *Nature* **387,** 299–303
14. Honda, R., Tanaka, H., and Yasuda, H. (1997) Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. *FEBS Lett.* **420,** 25–27
15. Piette, J., Neel, H., and Maréchal, V. (1997) Mdm2. Keeping p53 under control. *Oncogene* **15,** 1001–1010
16. Yuan, Z. M., Huang, Y., Ishiko, T., Nakada, S., Utsugisawa, T., Shioya, H., Utsugisawa, Y., Yokoyama, K., Weichselbaum, R., Shi, Y., and Kufe, D. (1999) Role for p300 in stabilization of p53 in the response to DNA damage. *J. Biol. Chem.* **274,** 1883–1886
17. Oren, M. (2003) Decision making by p53. Life, death, and cancer. *Cell Death Differ.* **10,** 431–442
18. D'Orazi, G., Cecchinelli, B., Bruno, T., Manni, I., Higashimoto, Y., Saito, S., Gostissa, M., Coen, S., Marchetti, A., Del Sal, G., Piaggio, G., Fanciulli, M., Appella, E., and Soddu, S. (2002) Homeodomain-interacting protein kinase-2 phosphorylates p53 at Ser 46 and mediates apoptosis. *Nat. Cell Biol.* **4,** 11–19
19. Yoshida, K., Liu, H., and Miki, Y. (2006) Protein kinase Cδ regulates Ser^{46} phosphorylation of p53 tumor suppressor in the apoptotic response to DNA damage. *J. Biol. Chem.* **281,** 5734–5740
20. Oda, K., Arakawa, H., Tanaka, T., Matsuda, K., Tanikawa, C., Mori, T., Nishimori, H., Tamai, K., Tokino, T., Nakamura, Y., and Taya, Y. (2000) p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. *Cell* **102,** 849–862
21. Shikama, N., Lee, C. W., France, S., Delavaine, L., Lyon, J., Krstic-Demonacos, M., and La Thangue, N. B. (1999) A novel cofactor for p300 that regulates the p53 response. *Mol. Cell* **4,** 365–376
22. Lacroix, M., Toillon, R. A., and Leclercq, G. (2006) p53 and breast cancer, an update. *Endocr. Relat. Cancer* **13,** 293–325
23. Kim, W. J., Rivera, M. N., Coffman, E. J., and Haber, D. A. (2012) The WTX tumor suppressor enhances p53 acetylation by CBP/p300. *Mol. Cell* **45,** 587–597
24. Ivanov, G. S., Ivanova, T., Kurash, J., Ivanov, A., Chuikov, S., Gizatullin, F., Herrera-Medina, E. M., Rauscher, F., 3rd, Reinberg, D., and Barlev, N. A. (2007) Methylation-acetylation interplay activates p53 in response to DNA damage. *Mol. Cell. Biol.* **27,** 6756–6769
25. Samuels-Lev, Y., O'Connor, D. J., Bergamaschi, D., Trigiante, G., Hsieh, J. K., Zhong, S., Campargue, I., Naumovski, L., Crook, T., and Lu, X. (2001) ASPP proteins specifically stimulate the apoptotic function of p53. *Mol. Cell* **8,** 781–794
26. Yang, H. Y., Wen, Y. Y., Chen, C. H., Lozano, G., and Lee, M. H. (2003) 14-3-3 sigma positively regulates p53 and suppresses tumor growth. *Mol. Cell. Biol.* **23,** 7096–7107
27. Ito, Y. (2008) RUNX genes in development and cancer. Regulation of viral gene expression and the discovery of RUNX family genes. *Adv. Cancer Res.* **99,** 33–76
28. Miyoshi, H., Shimizu, K., Kozu, T., Maseki, N., Kaneko, Y., and Ohki, M. (1991) t(8;21) breakpoints on chromosome 21 in acute myeloid leukemia are clustered within a limited region of a single gene, *AML1*. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 10431–10434
29. Golub, T. R., Barker, G. F., Bohlander, S. K., Hiebert, S. W., Ward, D. C., Bray-Ward, P., Morgan, E., Raimondi, S. C., Rowley, J. D., and Gilliland, D. G. (1995) Fusion of the TEL gene on 12p13 to the *AML1* gene on 21q22 in acute lymphoblastic leukemia. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 4917–4921
30. Silva, F. P., Morolli, B., Storlazzi, C. T., Anelli, L., Wessels, H., Bezrookove, V., Kluin-Nelemans, H. C., and Giphart-Gassler, M. (2003) Identification of RUNX1/AML1 as a classical tumor suppressor gene. *Oncogene* **22,** 538–547
31. Roumier, C., Fenaux, P., Lafage, M., Imbert, M., Eclache, V., and Preudhomme, C. (2003) New mechanisms of *AML1* gene alteration in hematological malignancies. *Leukemia* **17,** 9–16
32. Okuda, T., van Deursen, J., Hiebert, S. W., Grosveld, G., and Downing, J. R. (1996) AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. *Cell* **84,** 321–330
33. Wang, Q., Stacy, T., Binder, M., Marin-Padilla, M., Sharpe, A. H., and Speck, N. A. (1996) Disruption of the *Cbfa2* gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 3444–3449
34. Takahashi, A., Satake, M., Yamaguchi-Iwai, Y., Bae, S. C., Lu, J., Maruyama, M., Zhang, Y. W., Oka, H., Arai, N., Arai, K., *et al.* (1995) Positive and negative regulation of granulocyte-macrophage colony-stimulating factor promoter activity by AML1-related transcription factor, PEBP2. *Blood* **86,** 607–616
35. Taniuchi, I., Osato, M., Egawa, T., Sunshine, M. J., Bae, S. C., Komori, T., Ito, Y., and Littman, D. R. (2002) Differential requirements for Runx proteins in CD4 repression and epigenetic silencing during T lymphocyte development. *Cell* **111,** 621–633
36. Lutterbach, B., Westendorf, J. J., Linggi, B., Isaac, S., Seto, E., and Hiebert, S. W. (2000) A mechanism of repression by acute myeloid leukemia-1, the target of multiple chromosomal translocations in acute leukemia. *J. Biol. Chem.* **275,** 651–656
37. Guo, H., and Friedman, A. D. (2011) Phosphorylation of RUNX1 by cyclin-dependent kinase reduces direct interaction with HDAC1 and HDAC3. *J. Biol. Chem.* **286,** 208–215
38. Yamaguchi, Y., Kurokawa, M., Imai, Y., Izutsu, K., Asai, T., Ichikawa, M., Yamamoto, G., Nitta, E., Yamagata, T., Sasaki, K., Mitani, K., Ogawa, S., Chiba, S., and Hirai, H. (2004) AML1 is functionally regulated through p300-mediated acetylation on specific lysine residues. *J. Biol. Chem.* **279,** 15630–15638
39. Zhao, X., Jankovic, V., Gural, A., Huang, G., Pardanani, A., Menendez, S., Zhang, J., Dunne, R., Xiao, A., Erdjument-Bromage, H., Allis, C. D., Tempst, P., and Nimer, S. D. (2008) Methylation of RUNX1 by PRMT1 abrogates SIN3A binding and potentiates its transcriptional activity. *Genes Dev.* **22,** 640–653
40. Linggi, B., Müller-Tidow, C., van de Locht, L., Hu, M., Nip, J., Serve, H., Berdel, W. E., van der Reijden, B., Quelle, D. E., Rowley, J. D., Cleveland, J., Jansen, J. H., Pandolfi, P. P., and Hiebert, S. W. (2002) The t(8;21) fusion protein, AML1 ETO, specifically represses the transcription of the p14(ARF) tumor suppressor in acute myeloid leukemia. *Nat. Med.* **8,** 743–750
41. Wotton, S. F., Blyth, K., Kilbey, A., Jenkins, A., Terry, A., Bernardin-Fried, F., Friedman, A. D., Baxter, E. W., Neil, J. C., and Cameron, E. R. (2004) RUNX1 transformation of primary embryonic fibroblasts is revealed in the absence of p53. *Oncogene* **23,** 5476–5486
42. Li, X. L., Arai, Y., Harada, H., Shima, Y., Yoshida, H., Rokudai, S., Aikawa, Y., Kimura, A., and Kitabayashi, I. (2007) Mutations of the *HIPK2* gene in acute myeloid leukemia and myelodysplastic syndrome impair AML1- and p53-mediated transcription. *Oncogene* **26,** 7231–7239
43. Hanamoto, T., Ozaki, T., Furuya, K., Hosoda, M., Hayashi, S., Nakanishi, M., Yamamoto, H., Kikuchi, H., Todo, S., and Nakagawara, A. (2005) Identification of protein kinase A catalytic subunit beta as a novel binding partner of p73 and regulation of p73 function. *J. Biol. Chem.* **280,** 16665–16675
44. Li, Y., Ozaki, T., Kikuchi, H., Yamamoto, H., Ohira, M., and Nakagawara, A. (2008) A novel HECT-type E3 ubiquitin protein ligase NEDL1 enhances the p53-mediated apoptotic cell death in its catalytic activity-independent manner. *Oncogene* **27,** 3700–3709

45. Yoshihara, Y., Wu, D., Kubo, N., Sang, M., Nakagawara, A., and Ozaki, T. (2012) Inhibitory role of E2F-1 in the regulation of tumor suppressor p53 during DNA damage response. *Biochem. Biophys. Res. Commun.* **421,** 57–63
46. Kitabayashi, I., Yokoyama, A., Shimizu, K., and Ohki, M. (1998) Interaction and functional cooperation of the leukemia-associated factors AML1 and p300 in myeloid cell differentiation. *EMBO J.* **17,** 2994–3004
47. Sakaguchi, K., Herrera, J. E., Saito, S., Miki, T., Bustin, M., Vassilev, A., Anderson, C. W., and Appella, E. (1998) DNA damage activates p53 through a phosphorylation-acetylation cascade. *Genes Dev.* **12,** 2831–2841
48. Tang, Y., Zhao, W., Chen, Y., Zhao, Y., and Gu, W. (2008) Acetylation is indispensable for p53 activation. *Cell* **133,** 612–626
49. Michishita, E., Park, J. Y., Burneskis, J. M., Barrett, J. C., and Horikawa, I. (2005) Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins. *Mol. Biol. Cell* **16,** 4623–4635
50. Wen, Z., Pyeon, D., Wang, Y., Lambert, P., Xu, W., and Ahlquist, P. (2012) Orphan nuclear receptor PNR/NR2E3 stimulates p53 functions by enhancing p53 acetylation. *Mol. Cell. Biol.* **32,** 26–35
51. Kawai, H., Nie, L., Wiederschain, D., and Yuan, Z. M. (2001) Dual role of p300 in the regulation of p53 stability. *J. Biol. Chem.* **276,** 45928–45932
52. Zhao, Y., Lu, S., Wu, L., Chai, G., Wang, H., Chen, Y., Sun, J., Yu, Y., Zhou, W., Zheng, Q., Wu, M., Otterson, G. A., and Zhu, W. G. (2006) Acetylation of p53 at lysine 373/382 by the histone deacetylase inhibitor depsipeptide induces expression of p21(Waf1/Cip1). *Mol. Cell. Biol.* **26,** 2782–2790
53. Wildey, G. M., Patil, S., and Howe, P. H. (2003) Smad3 potentiates transforming growth factor β (TGFβ)-induced apoptosis and expression of the BH3-only protein Bim in WEHI 231 B lymphocytes. *J. Biol. Chem.* **278,** 18069–18077
54. Wildey, G. M., and Howe, P. H. (2009) Runx1 is a co-activator with FOXO3 to mediate transforming growth factor β (TGFβ)-induced Bim transcription in hepatic cells. *J. Biol. Chem.* **284,** 20227–20239
55. Yano, T., Ito, K., Fukamachi, H., Chi, X. Z., Wee, H. J., Inoue, K., Ida, H., Bouillet, P., Strasser, A., Bae, S. C., and Ito, Y. (2006) The RUNX3 tumor suppressor up-regulates Bim in gastric epithelial cells undergoing transforming growth factor β-induced apoptosis. *Mol. Cell. Biol.* **26,** 4474–4488
56. Yamada, C., Ozaki, T., Ando, K., Suenaga, Y., Inoue, K., Ito, Y., Okoshi, R., Kageyama, H., Kimura, H., Miyazaki, M., and Nakagawara, A. (2010) RUNX3 modulates DNA damage-mediated phosphorylation of tumor suppressor p53 at Ser-15 and acts as a co-activator for p53. *J. Biol. Chem.* **285,** 16693–16703

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A *MYCN*-amplified cell line derived from a long-term event-free survivor among our sixteen established neuroblastoma cell lines

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ABSTRACT

Although more than 110 neuroblastoma (NB) cell lines have been established, there have been neither reports on the rate of success to establish NB cell lines, nor well-documented NB cell lines from long-term-survivors. We attempted to establish NB cell lines from 114 patients. Sixteen NB cell lines were established from 12 patients. The success rates to establish cell lines were 1.4% (1/70) from patients in early stages, 25.0% (11/44) from those in advanced stages, and 10.5% (12/114) from those in all stages respectively. Eleven of these 12 patients eventually died. The surviving patient, who was in stage 4 with *MYCN*-amplification, has been event-free for 19 years after completing therapy. The serum *MYCN* DNA level in patient TK was very high before therapy, decreased after chemotherapy, and has remained at the normal levels until now. The gene expression profiling of the primary tumor and the K-N-TK cell line was analyzed with an NB-specific cDNA microarray, and indicated that the probability of 5-year survival was extremely low. Microarray-based comparative genomic hybridization (CGH) analysis indicated that genomic aberration profiles of the cell line were uncommon, with *MYCN* amplification, 17q gain and 11q loss. A unique KP-N-TK cell line, established from an event-free survivor, will be a useful tool for investigating how a patient can survive a tumor with an extremely poor prognosis.

1. Introduction

Neuroblastoma (NB) is a common type of malignant solid tumor in childhood, arising from neural crest precursors. The first NB cell lines, NB-1 and SK-N-SH, were established in 1973 [1,2]. Since then, more than 110 NB cell lines have been reported [3]. Most NB cell lines were generated from tumor samples obtained from patients with an advanced stage of neoplastic disease.

NB cells to grow progressively in a tissue culture flask enable us to establish a permanent cell line, and the inverse correlation with the survival rate of patients, from whom the NB cell lines were derived, was reported [3,4].

This study was undertaken to estimate the success rate to establish NB cell lines from 114 patients with early or advanced stages, to know the patient's outcomes whose cell lines were generated. For the KP-N-TK cell line, established from a long-term survivor of patient TK, we assayed *MYCN* DNA levels of primary tumor cells, the cell line and serum levels before and after therapy to monitor the residual tumor cells during the patient's clinical course. Finally, microarray analyses of both gene expression profiles and genomic DNA aberrations in primary tumor cells of patient TK and/or the KP-N-TK cell line were analyzed to see if they could explain patient TK's long-term survival.

2. Materials and methods

2.1. Patients from whom we attempted to establish NB cell lines

The patients at the Hospital of Kyoto Prefectural University of Medicine, University of Miyazaki, and the affiliated hospitals in Japan, were enrolled in this study with the informed consent of their parents. The study was conducted under research protocols approved by the institutional review boards. From 1983 to 2005 we attempted to establish NB cell lines from 114 patients (37 patients with stage 1 NB, 26 stage 2, 9 stage 3, 35 stage 4, and 7 stage 4S). NB was detected in 67 of these patients by the Japanese Mass Screening System (Table 1).

2.2. Clinical outcome of 114 patients and clinical history of patient TK

Five-year overall-survivals (OSs) were high in the early stages (100% in stage 1, 96.1% in stage 2 and 85.7% in stage 4S respectively) and low in advanced stages (55.5% in stage 3 and 25.7% in stage 4 respectively) (Table 1). Among the 114

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attempts to establish an NB cell line, only one was successful. The source of the cell line was a long-term survivor of NB, a Japanese male referred to as patient TK. In February 1992, at age 13 months, he presented with anemia and right-exophthalmos. Clinical examination revealed a left upper abdominal mass, and magnetic resonance imaging showed a primary abdominal tumor arising from the left adrenal gland and a right orbital bone metastasis. Morphological and surface membrane analysis [5] of bone marrow aspirates revealed that most (80%) of the normal hematopoietic progenitor cells had been replaced by tumor. The right orbital bone and bone marrow metastases led to NB with stage 4. High levels of the catecholamine metabolites, vanillylmandelic acid (VMA) and homovanillic acid (HVA), were identified in the patient's urine.

Biopsy of the primary tumor prior to chemotherapy showed small-to medium-sized round tumor cells with poor stromal development and a high mitosis–karyorrhexis index (MKI) (437/5000) (Fig. 1). The tumor was diagnosed as an unfavorable type of NB (Schwannian stroma-poor, undifferentiated subtype with high MKI) according to the International NB Pathology Classification [6].

The patient was treated with six courses of chemotherapy consisting of a combination of cyclophosphamide, etoposide, doxorubicin and cisplatinum, followed by a delayed-primary operation. Complete remission of primary and metastatic lesions was confirmed by magnetic resonance imaging, MIBG scintigraphy, bone marrow aspiration and urinary VMA and HVA levels. He was then given megatherapy with peripheral blood stem cell transplantation (PBSCT) [7,8]. Treatment was terminated in April 1993, and the patient has been event-free for 19 years after completing therapy.

2.3. Cell culture to establish NB cell lines

Tumor samples from the 114 patients (Table 1) for cell culture were obtained from biopsy, operation or autopsy and finely minced with scalpels and cultured. For patient TK, prior to chemotherapy, primary tumor cells but not bone marrow tumor cells were obtained by biopsy in March 1992 and cultured. Mononuclear cell fractions of cells from bone marrow metastatic samples were prepared by Ficoll-Hypaque centrifugation. Cells were cultured in RPMI 1640 medium containing penicillin (100 U/ml), streptomycin (100 μg/ml) and 15% heated-inactivated fetal calf serum at 37 °C in 5% CO_2 in air. The medium was exchanged every 3–4 days [5]. Cell lines were considered to be established when they have been maintained for more than 60 passages over a 2-year period.

2.4. Identification of KP-N-TK cell line from patient TK

A Cell ID System (Promega, Madison, WI) based on short tandem repeats (STR) was used to compare KP-N-TK cells with WBC and cells from the primary tumor of patient TK [9]. Genomic DNA was used for PCR analyses. The tool of online verification of cell line is available from DSMZ Online STR Analysis (http://www.dsmz.de/services/services-human-and-animal-cell-lines/online-str-analysis.html). The identity of two cell lines (A and B) is expressed as an evaluation value (EV) calculated as EV = {Number of coincident peaks of STR profiles between A and B} × 2/{Total number of peaks of STR profiles in A and B}. EV values greater than 0.9 indicate that the two cell types are derived from the same origin.

2.5. Assay of tumor and serum MNA by real-time quantitative PCR

MYCN amplification (MNA) in the original tumor sample and KP-N-TK cell line was determined by our developed real-time quantitative PCR [10] instead of the conventional Southern blot analysis [11,12]. DNA was prepared from tumor tissue, cell line and stored serum (200 μL). The *MYCN* (2p24) copy number of a sample of DNA was determined by the ratio of the *MYCN* dosage to the *N-acetylglucosamine kinase gene* (*NAGK*) (2p12) dosage (*M*/*N* ratio). This method has several advantages over FISH and Southern blotting methods, including short turnaround time (4 h), much less effort, well-correlated *M*/*N* ratio between tumor DNA and serum DNA, and more highly accurate detection of MNA gene status. Another clinical benefit of the serum *M*/*N* assay is that the *M*/*N* ratio can be used as a tumor marker [10].

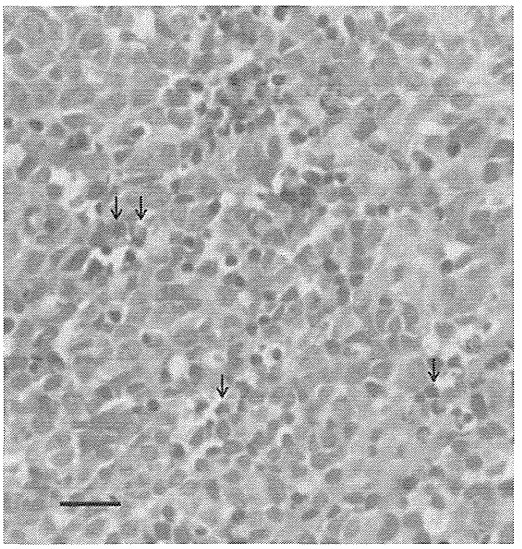

Fig. 1. Histological appearance of the primary tumor of patient TK on biopsy. Schwannian stroma-poor, undifferentiated medium to large size tumor cells were seen. The mitosis–karyorrhexis index (MKI), indicated by arrows, was very high (437/5,000). Scale bar: 20 μm.

2.6. Gene expression profiling of the tumor by the NB-proper cDNA microarray

We previously constructed an NB diagnosis mini-chip which harbors the 200 top-ranked prognosis-related genes and developed a computational algorithm for prognosis prediction by using the 50 gene profiles in the tumor (Table 1S).

The survival probability of each patient at 5 years after diagnosis was calculated by the computational algorithm as a posterior value (ranging from 0 to 1) [13,14].

2.7. Microarray-based comparative genomic hybridization of KP-N-TK cell line

DNA copy number alterations in the tumor were determined with an array-based CGH using a DNA chip carrying 2464 BAC clones (Agilent Technology, Santa Clara, CA) as previously reported [14,15]. The KP-N-TK cell line (60 passages, January 1995) was used for this assay because the amount of primary tumor cells was insufficient.

3. Results

3.1. Establishment of NB cell lines

NB cell lines were identified as NBs from the clinical features of the patients, surface-membrane analysis with a panel of nine monoclonal antibodies [5], and by a cytoskeletal protein analysis with anti-neurofilament antibody [16,17], as previously reported.

From 114 patients with NB, 16 NB cell lines, derived from 12 patients, were established.

The success rate of establishing cell lines was 0% (0/37 patients) in stage 1, 0% (0/26) in stage 2, 11.1% (1/9) in stage 3, 28.6% (10/35)

Table 1
Effect of NB stage on the 5-year over-all survival, success rate of establishing NB cell lines from 114 patients.

Stage	Primary culture	Establishment of cell line	
	Survival pt[a]/total pt (5-year OS[b] %)	Success rate (%)	Survival pt/pt established cell line (5-year OS %)
Stage 1	37/37 (100)	0/37 (0)	0/0 (0)
Stage 2	25/26 (96.1)	0/26 (0)	0/0 (0)
Stage 3	5/9 (55.5)	1/9 (11.1)	0/1 (0)
Stage 4	9/35 (25.7)	10/35 (28.6)	1/10 (10)
Stage 4S	6/7 (85.7)	1/7 (14.3)	0/1 (0)
Total	82/114 (71.9)	12/114 (10.5)	1/12 (8.3)

The success rate of establishing cell lines from early (1, 2 and 4S) stage was 1.4% (1patient/70 patients), and from advanced (3 and 4) stage was 25.0% (11/44).
[a] Patient.
[b] Overall-survival.

in stage 4, 14.3% (1/7) in stage 4S and 10.5% (12/114) in all stages, respectively (Table 1). Five-year OS rate (%) among 12 patients, from whose cell lines were established, was 0% (0/1 patient) in stage 3, 10% (1/10) in stage 4, 0% (0/1) in stage 4S and 8.3% (1/12) in all stages (Table 1).

Table 2 lists the characteristics of 16 NB cell lines from 12 patients in our laboratories. Ten of the samples (62.5%) used to establish these cell lines were collected before any therapy, four samples (25.0%) were collected during chemotherapy, and two samples (12.5%) were collected at autopsy. Eleven cell lines were from NBs with MNA and 5 from NBs without MNA. Of these 12 patients, 11 patients eventually died and only one patient (patient No. 12) survived (Table 2) [5,11,12,17–28].

3.2. Establishment of the KP-N-TK NB cell line

Minced tumor cells obtained from a biopsy in March 1992 (before therapy) grew in the form of adherent cells. The cells were spindle-shaped or focally aggregated with neurite processes, and were maintained for more than 60 passages. The cell line, designated as KP-N-TK, was established in January 1995.

3.3. Confirmation that the KP-N-TK ell line was derived from patient TK

STR analysis of primary TK tumor and the KP-N-TK cell line was identical. STR analysis yielded an EV between both primary TK tumor (sampled March 1992) and the KP-N-TK cell line (July 1999), and WBC (November 2009) from patient TK of 0.966, indicating that the three cell types had the same origin. STR analysis of the primary TK tumor and the KP-N-TK cell line revealed a loss of heterozygosity (LOH) at D16S539 (16q24-qter) and Amelogenin X (Xp22.1–22.3, Y) (Table 3). The LOH results were in agreement with those of CGH described below, revealing whole losses of 16q and X (data not shown).

Table 2
Characteristics of 16 NB cell lines established from 12 patients by our laboratories.

Cell line	Patient number[a]	Age y m[b]	Primary tumor	Stage	Metastasis	Sample	Therapy	*MYCN* amplification[c]	Outcome	Refs.
KP-N-RT-BM	01	1 y 2 m	Adr gl[d]	4	BM[e], Bone	BM	Before[f]	50	Dead	[5,12,17–19]
KP-N-RT-LN	01	1 y 2 m	Adr gl	4	BM, Bone, LN[g]	LN	Before	50	Dead	[5,11,20,21]
KP-N-RT-BMV	01	1 y 2 m	Adr gl	4	BM, Bone	BM	Before	100	Dead	[12]
KP-N-SILA	02	5 y	Adr gl	4S	LN, Bone	LN	Autopsy[h]	1	Dead	[16,17,22]
KP-N-SIFA	02	5 y	Adr gl	4S	LN, Bone	Bone	Autopsy	1	Dead	[17–19,23,24]
KP-N-YN	03	2 y	Adr gl	3	LN	Delayed primary[i]	During[j]	100	Dead	[16,17]
KP-N-AY	04	2 y 6 m	Adr gl	4	LN, BM	BM	Before	50	Dead	[25]
KP-N-AYR	04	2 y 6 m	Adr gl	4	LN, BM	BM	During	50	Dead	[25]
MP-N-MS	05	1 y 6 m	Adr gl	4	BM	BM	Before	50	Dead	[17]
KP-N-YS	06	4 y	Adr gl	4	BM	BM	Before	10	Dead	[17]
MP-N-TS	07	2 y 8 m	Adr gl	4	Bone, Gingiva	Primary[k]	Before	1	Dead	[26–28]
KP-N-HN	08	4 y	Adr gl	4	BM	BM	During	1	Dead	[26]
KP-N-NY	09	5 y	Adr gl	4	BM	Delayed primary	During	1	Dead	[26]
KP-N-SK	10	2 y 2 m	Adr gl	4	Bone, BM	BM	Before	45	Dead	Unpublished
KP-N-YuNo	11	1 y 5 m	Retro[l]	4	LN	Primary	Before	10	Dead	Unpublished
KP-N-TK	12	1 y 1 m	Adr gl	4	BM, Bone	Primary	Before	20	Alive	[23,24]

Sixteen NB cell lines were established from 12 patients. KP-N-TK cell line is the only cell line whose patient had a long-term (19-year) event-free survival.
[a] Patient number: A same number indicates the established cell line from the same patient.
[b] y m: year month.
[c] MYCN amplicication: MYCN copy number by Southern blot.
[d] Adr gland: adrenal gland.
[e] BM: bone marrow.
[f] Before: Sample obtained before any therapy.
[g] LN: lymph node.
[h] Autopsy: Sample obtained at autopsy on tumor death.
[i] Delayed primary: delayed primary tumor.
[j] During: Sample obtained during therapy.
[k] Primary: primary tumor.
[l] Retro: retroperitoneal.

Table 3
STR analysis of primary TK tumor, KP-N-TK cell line and WBC from patient TK.

STR	Locus	Primary TK tumor		KP-N-TK cell line		WBC from patient TK	
		Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2
TPOX	2p23–2pter	8	11	8	11	8	11
D5S818	5q23.3–32	8	12	8	12	8	12
CSF1PO	5q33.3–34	11	12	11	12	11	12
D7S820	7q11.21–22	11	–	11	–	11	–
TH01	11p15.5	7	9	7	9	7	9
vWA	12p12-pter	17	–	17	–	17	–
D13S317	13q22-q31	9	11.2	9	11.2	9	11.2
D16S539	16q24-qter	11	–	11	–	9	11
D21S11	21q11–21q21	29	–	29	–	29	–
Amelogenin X	Xp22.1–22.3 and Y	X	–	X	–	X	Y

STR analysis of primary TK tumor, KP-N-TK cell line and WBC from patient TK indicated that the three cell types had the same origin.

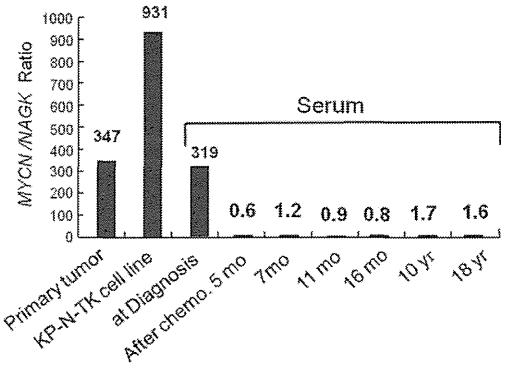

Fig. 2. *MYCN* DNA levels of primary TK tumor, KP-N-TK cell line and serum before and after chemotherapy by quantitative PCR.

3.4. MYCN DNA levels of primary tumor, cell line and serum

The *MYCN* DNA levels, expressed as *M/N* ratios, in the primary tumor and serum at diagnosis were very similar (347 and 319, respectively). The *M/N* ratio in KP-N-TK cells at passage 70 (931) was 2.7 times higher level than that of the primary tumor (347), suggesting the selection of *MYCN*-amplified clone during the culture period. In patient TK, the serum *MYNC* level was 319 at diagnosis in March 1992, decreased to 0.6 in September 1993 after 5 months of chemotherapy, and has consistently remained low (below 2.2) for the last 18 years (Fig. 2).

3.5. Gene expression signatures of primary TK tumor and the KP-N-TK cell line

The gene expression profiles of primary tumor tissue obtained in March 1992 and KP-N-TK cells from passage 62 were determined with a microarray spotted with the 200 top-ranked genes for clinical use (Table 1S) [13,14]. Four of nine genes associated with a poor prognosis were strongly expressed, and five of seven genes associated with a favorable prognosis were weakly expressed in both the primary TK tumor and KP-N-TK cells. The unfavorable prognosis genes were ribosomal protein genes *RPL18A* (Table 1S, Gene No. 2) and *RPLP0* (Gene No. 24), translation initiation gene *EEF1G* (Gene Nos. 3 and 22) and metabolism gene *enolase 1* (Gene No. 10). The favorable prognosis genes were neuronal differentiation genes *tubulin α* (Gene No. 46), *peripherin* (Gene Nos. 38 and 50), *neuromodulin* {*GAP43*}(Gene No. 44), and *HMP19* (Gene No. 42) and catecholamine metabolism gene *tyrosine hydroxylase* {*TH*} (Gene No. 28) (Fig. 3).

With regard to the *MYCN* expression we have a set of expression data obtained from 50 reference samples (41 non-*MYCN* amplified and 9 *MYCN*-amplified) by the microarray carrying the 200 top-ranked genes for clinical use (Fig. 3) [13]. Average log2 expression ratios in tumors with and without MNA were 1.243 and -0.015, respectively. In contrast, those of primary TK tumor and the KP-N-TK cell line were 0.312 and 1.046, respectively (average value of Gene No. 7 and Gene No. 9 in Fig. 3). The expression levels of *MYCN* of the primary TK tumor and the KP-N-TK cell line were less than those in typical *MYCN*-amplified tumors, and the expression levels in the cell line were higher than those in primary tumor cells.

The expression level of *NTRK1* (*TRKA*) expression (Gene No. 47) was moderate in both samples (Fig. 3).

The posterior values of primary TK tumor and the KP-N-TK cell line at 5 years after diagnosis were 0.003 and 0.001, respectively, showing that the prognosis of patient TK, based on the mini-chip algorithm, was extremely poor [13,14] .

3.6. Genomic aberration signature of KP-N-TK cell line

The array CGH analysis indicated that the KP-N-TK cell line had multiple chromosomal aberrations (data not shown). Based on four of these characteristics (chromosome 1 without deletion of 1p36, chromosome 2 with MNA at 2p24, chromosome 11 with deletion of 11q23 and chromosome 17 with 17q21-qter gain), the KP-N-TK cell line was classified as a GGP3a tumor which is an uncommon feature among NB tumors [14,15].

4. Discussion

NB cell lines are some of the first and most widely used human tumor cell lines. More than 110 established NB cell lines have been reported in a review [3]. Our cell lines, like most other NB cell lines [3,4], were established from patients with advanced stage diseases. Our established cell lines have been used for many studies, including studies of neuronal or schwannian cell differentiation [11,12,18–21], cell surface membrane analysis by monoclonal antibodies [5], S-type cells expressing smooth-muscle-cell phenotypes [16,17], drug resistance [25], signal transduction of neurotrophic-factors [18,26,27], signal transduction on apoptosis [23,24], and molecular biology of oncogenes including MNA [11,12,28] (Table 2).

An accurate success rate for establishing NB cell lines from primary cell cultures by using the identical procedure like in our laboratories has never been reported. Our success rate to establish cell lines from early (1, 2 and 4S) stage was 1.4% (1/70 patients). That is, the KP-N-SILA and KP-N-SIFA cell lines [16–19,22–24] were established from only one patient SI with favorable stage 4S among 67 patients detected by the Japanese Mass Screening System. In contrast, the success rates of cells from advanced (3 and 4) stage was 25.0% (11/44 patients), which were higher than expected (Table 1). The prognosis of patients whose NB cell lines are established is reported to be extremely poor [3,4]. Although a few personal communications are available, no well-documented NB cell lines with long-term survivors have been reported. Among our 12 patients whose cell lines were established, only patient TK survived.

Recently the International Neuroblastoma Risk Group (INRG) classification system was developed to establish a consensus approach for pretreatment risk stratification by the statistically significant and clinically relevant factors [29]. Of those the 5-year event-free survival (EFS) rate of patient TK by nine potential prognostic factors were: 35% based on his INSS stage (stage 4), 49% based on his age (over 365 days), 40% based on histological classification (unfavorable), 63% based on grade of NB differentiation (undifferentiated), 37% based on high MKI, 26% based on MNA status, 55% based on ploidy (diploid), 35% based on 11q status (aberration), and 41% based on 17q gain respectively. All of these factors except for tumor differentiation and ploidy had predicted 5-year EFSs of less than 50%, putting patient TK in a high risk group.

MNA is strongly associated with rapid tumor progression, which makes MNA the most powerful prognostic factor. The inverse relation between number of *MYCN*-gene copies and progression-free survival was significant among patients with stage 4 [30].

We previously reported a quantitative PCR method for measuring circulatory and tumor *MYCN* copy number [10]. This method has several advantages over FISH and Southern blotting methods as described in Materials and Methods. The higher *MYCN* status in primary tumor obtained by quantitative PCR (Fig. 2; *M/N* ratio = 347) is more accurate than the *MYCN* status obtained by Southern blotting (21 copies as compared with the single copy number control tissue of human placental DNA) (data not shown) [11,12]. It also shows a poorer prognosis for patient TK than the *MYCN* status estimated by Southern blotting.

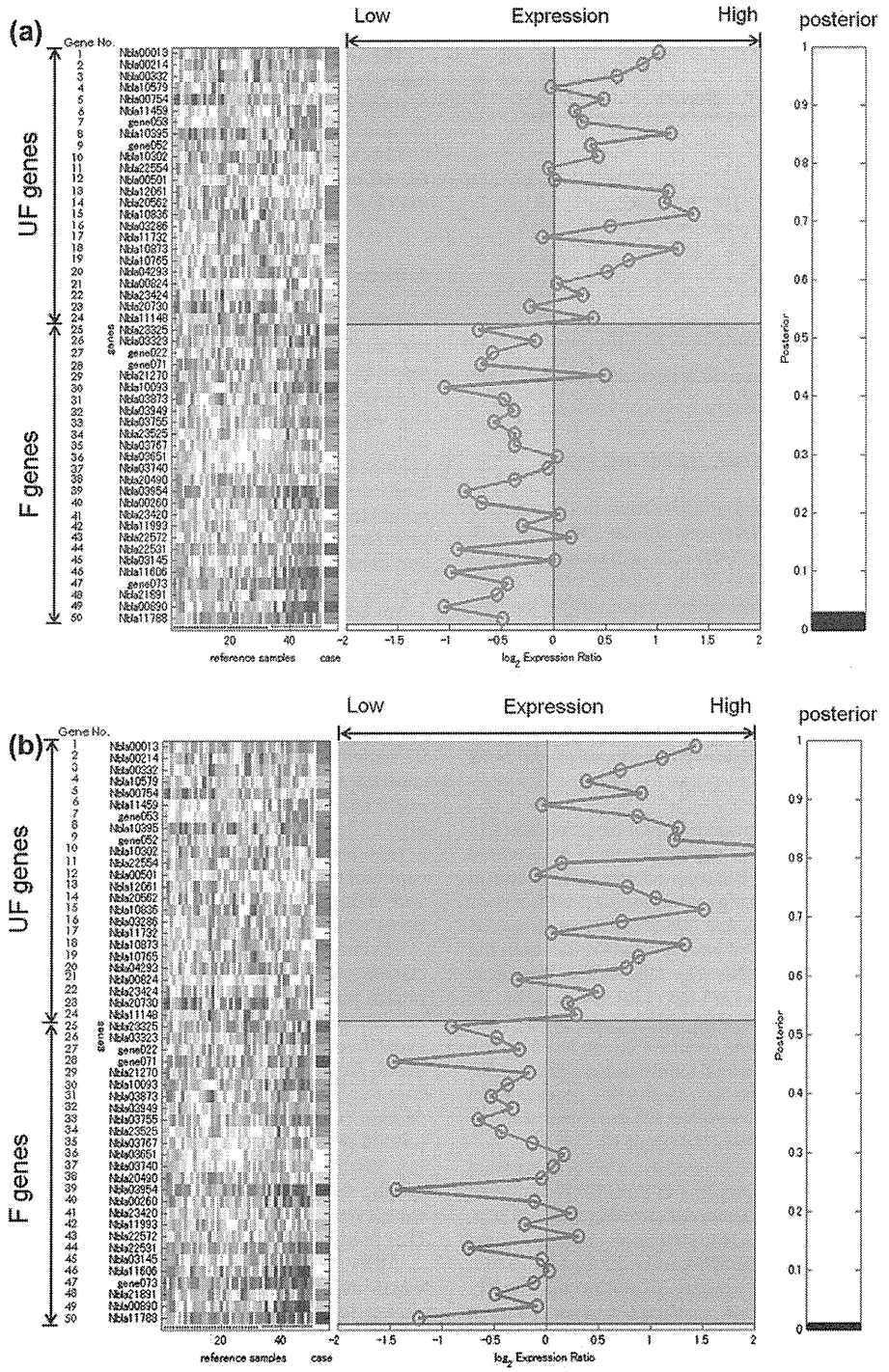

Fig. 3. Gene expression signatures of selected 50 genes in primary TK tumor and KP-N-TK cell line for predicting NB prognosis. (**a**) Primary TK tumor and (**b**) KP-N-TK cell line Twenty-four genes related to unfavorable prognosis (UF genes) and 26 genes related to favorable prognosis (F genes), listed in Table 1S, were analyzed. From left to right panels, gene number (n = 50), gene ID, gene expression profile of 50 reference samples, gene expression profile of primary TK tumor or KP-N-TK cell line, and posterior value are shown. The blue and red colors in the expression matrix show the high and low expression, respectively.

The clinical benefit of the serum *M*/*N* assay is that the *M*/*N* ratio can be used as a tumor marker [10]. The prognosis of patient TK was predicted to be very poor based on the cell line success and the INRG classification as described above. The *MYCN* copy number in human NB is usually consistent within a tumor, not only at different tumor sites, but also at different times in clinical courses

[31]. Therefore, serial serum levels of *MYCN* DNA in patient TK consequently demonstrated that the eradication of residual NB cells and the chemosensitive TK tumor cells by the chemotherapy with PBSCT. Thus, this is the first report of long-term (18-year) monitoring of the serum *M*/*N* ratio as a potent tumor maker before and after chemotherapy (Fig. 2).

In the primary tumor of patient TK and the KP-N-TK cell line, unfavorable prognosis genes (related to protein synthesis, and metabolism) were strongly expressed, whereas favorable prognosis genes (related to neuronal differentiation and catecholamine metabolism) were lowly expressed.

In the reference set of 50 tumors, 3 of 9 tumors with MNA and one of 41 tumors without MNA had lower and higher expression ratios of *MYCN* mRNA, respectively (Fig. 3), as compared to the primary TK tumor. The former three patients with MNA but lower expression of *MYCN* mRNA died of cancer within 2 years after diagnosis. Our previous expression profiling of 136 NB clearly showed that higher *MYCN* mRNA expression is one of the top-ranked poor prognosis-related genes as well as MNA [13], however, we do not have enough evidence so far, due to the small number of such cases, to conclude that low *MYCN* mRNA expression, despite of MNA, could explain long-term survivors.

The expression profiles of the TK tumor and the KP-N-TK cell line were similar, suggesting that the cell line had maintained the gene expression profile of the tumor. Posterior value is the probability of survival at 5-years after diagnosis calculated from these two gene expression profiles, predicting the possibility of survival of patient TK was nearly 0% (Fig. 3) [13,14].

Our CGH studies indicated that the KP-N-TK cells had MNA, 11q loss and 17q gain, but not 1p loss, and were distinguished from other NBs and were classified into uncommon subgroup of GGP3a, among GGP tumor groups ($n = 77$) with partial chromosomal gains/losses. Among 236 samples examined, only one death patient had GGP3a tumor, so it was difficult to define the 5-year OS rate for this genomic group. The 5-year OS rate of 15 patients with GGP3s tumor, with single copy of *MYCN*, 11q loss and 17q gain, was 59%. On the other hand, GGP1a ($n = 22$), with MNA, 1p loss and 17q gain showed 44% of 5-year OS rate. Since the effect of 1p loss and 11q loss in the NB survival seems to be similar, GGP3a might be similar survival rate to those of GGP1a or GGP3s, although the sample number in GGP3a was very small [14,15].

The unexpected survival of patient TK might be caused by the very unfavorable gene expression (Fig. 3) and uncommon genomic aberration (MNA, 11q loss and 17q gain) of TK tumors and/or the KP-N-TK cell line which distinguished them from the other major *MYCN*-amplified tumors. Some specific biological and genetic characteristics of TK tumor cells related to growth, metastasis, survival, differentiation, apoptosis, chemosensitivity to anti-cancer drugs or host immune response may have affected on the prognosis of patient TK. New generation technologies such as whole genome SNP typing and sequencing, micro-RNA profiles and epigenetic modifications will help to understand the mechanisms involved in overcoming the poor prognosis of patients with *MYCN* amplified tumors.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.canlet.2012.12.011.

References

[1] S. Imashuku, A. Inui, T. Nakamura, J. Tanaka, S. Miyake, Catecholamine metabolism in tissue culture cells of a neuroblatoma, J. Clin. Endocrinol. Metab. 36 (1973) 931–936.
[2] J.L. Biedler, L. Helson, B.A. Spengler, Morphology and growth, tumorigenicity, and cytogenetics of human neuroblastoma cells in continuous culture, Cancer Res. 33 (1973) 2643–2652.
[3] C. Thiele, Neuroblastoma, in: J.R.W. Masters, B. Palsson (Eds.), Human Cell Culture, vol. 1, Kluwer Academic Publishers, Norwell, 1999, pp. 21–53.
[4] C.P. Reynolds, E.P. Frenkel, R.G. Smith, Growth characteristics of neuroblastoma in vitro correlate with patient survival, Trans. Assoc. Am. Phys. 93 (1980) 203–211.
[5] T. Sugimoto, T. Sawada, T. Matsumura, J.T. Kemshead, T. Ishii, Y. Horii, H. Morioka, M. Morita, C.P. Reynolds, Identical expression of cell surface membrane antigens on two parent and eighteen cloned cell lines derived from two different neuroblastoma metastases of the same patient, Cancer Res. 46 (1986) 4765–4769.
[6] H. Shimada, S. Umehara, Y. Monobe, Y. Hachitanda, A. Nakagawa, S. Goto, R.B. Gerbing, D.O. Stram, J.N. Lukens, K.K. Matthay, Cancer 92 (2001) 2451–2461.
[7] K. Kawa, N. Ohnuma, M. Kaneko, K. Yamamoto, T. Etoh, H. Mugishima, M. Ohhira, J. Yokoyama, F. Bessho, T. Honna, J. Yoshizawa, K. Nakada, M. Iwafuchi, T. Nozaki, J. Mimaya, T. Sawada, T. Nakamura, H. Miyata, K. Yamato, Y. Tsuchida, Long-term survivors of advanced neuroblastoma with MYCN amplification: a report of 19 patients surviving disease-free for more than 66 months, J. Clin. Oncol. 17 (1999) 3216–3220.
[8] M. Kaneko, Y. Tsuchida, H. Mugishima, N. Ohnuma, K. Yamamoto, K. Kawa, M. Iwafuchi, T. Sawada, S. Suita, Intensified chemotherapy increases the survival rates in patients with stage 4 neuroblastoma with *MYCN* amplification, J. Pediatr. Hematol. Oncol. 24 (2002) 613–621.
[9] W.G. Dirks, H.G. Drexler, Online verification of human cell line identity by STR DNA typing, in: I.A. Cree (Ed.), Cancer Cell Culture: Methods and Protocols, Methods in Molecular Biology, second ed., vol. 731, Springer Science, Berlin, 2011, pp. 45–55.
[10] T. Gotoh, H. Hosoi, T. Iehara, Y. Kuwahara, S. Osone, K. Tsuchiya, M. Ohira, A. Nakagawara, H. Kuroda, T. Sugimoto, Prediction of *MYCN* amplification in neuroblastoma using serum DNA and real-time quantitative polymerase chain reaction, J. Clin. Oncol. 23 (2005) 5205–5210.
[11] Y. Horii, T. Sugimoto, T. Sawada, J. Imanishi, K. Tsuboi, M. Hatanaka, Differential expression of *N-myc* and *c-src* proto-oncogenes during neuronal and schwannian differentiation of human neuroblastoma cells, Int. J. Cancer 43 (1989) 305–309.
[12] T. Hino, T. Sugimoto, T. Matsumura, Y. Horii, J. Inazawa, T. Sawada, Diverse responses to retinoid in morphological differentiation, tumorigenesis and *N-MYC* expression in human neuroblastoma sublines, Int. J. Cancer 44 (1989) 286–291.
[13] M. Ohira, S. Oba, Y. Nakamura, E. Isogai, S. Kaneko, A. Nakagawa, T. Hirata, H. Kubo, T. Goto, S. Yamada, Y. Yoshida, M. Fuchioka, S. Ishii, A. Nakagawara, Cancer Cell 7 (2005) 337–350.
[14] M. Ohira, A. Nakagawara, Global genomic and RNA profiles for novel risk stratification of neuroblastoma, Cancer Sci. 101 (2010) 2295–2301.
[15] N. Tomioka, S. Oba, M. Ohira, A. Misra, J. Fridlyand, S. Ishii, Y. Nakamura, E. Isogai, T. Hirata, Y. Yoshida, S. Todo, Y. Kaneko, D.G. Albertson, D. Pinkel, B.G. Feuerstein, A. Nakagawara, Oncogene 27 (2008) 441–449.
[16] T. Sugimoto, H. Ueyama, H. Hosoi, J. Inazawa, T. Kato, J.T. Kemshead, C.P. Reynolds, A.M. Gown, H. Mine, T. Sawada, Alpha-smooth-muscle actin and desmin expressions in human neuroblastoma cell lines, Int. J. Cancer 48 (1991) 277–283.
[17] T. Sugimoto, H. Mine, Y. Horii, K. Takahashi, R. Nagai, R. Morishita, M. Komada, Y. Asada, T. Sawada, Neuroblastoma cell lines showing smooth muscle cell phenotypes, Diagn. Mol. Pathol. 9 (2000) 221–228.
[18] A. Shikata, T. Shikata, Y. Sotozono, H. Hosoi H, T. Matsumura, T. Sugimoto, T. Sawada, Neuronal differentiation in human neuroblastoma cells by nerve growth factor following TrkA up-regulation by interferon-gamma, Med. Pediatr. Oncol. 34 (2000) 394–401.
[19] T. Sugimoto, Y. Horii, T. Hino, J.T. Kemshead, H. Kuroda, T. Sawada, H. Morioka, J. Imanishi, H. Inoko, Differential susceptibility of HLA class II antigens induced by gamma-interferon in human neuroblastoma cell lines, Cancer Res. 49 (1989) 1824–1828.
[20] T. Sugimoto, T. Sawada, T. Matsumura, Y. Horii, J.T. Kemshead, Y. Suzuki, M. Okada, O. Tagaya, T. Hino, Morphological differentiation of human neuroblastoma cell lines by a new synthetic polyprenoic acid (E5166), Cancer Res. 47 (1987) 5433–5438.
[21] T. Sugimoto, T. Kato, T. Sawada, Y. Horii, J.T. Kemshead, T. Hino, H. Morioka, H. Hosoi, Schwannian cell differentiation of human neuroblastoma cell lines *in vitro* induced by bromodeoxyuridine, Cancer Res. 48 (1988) 2531–2537.
[22] T. Matsumura, T. Gotoh, T. Sawada, T. Sugimoto, A neuroblastoma cell line derived from a case detected through a mass screening system in Japan: a case

report including the biologic and phenotypic characteristics of the cell line, Cancer 82 (1998) 1416–1417.
[23] S. Osone, H. Hosoi, Y. Kuwahara, Y. Matsumoto, T. Iehara, T. Sugimoto, Fenretinide induces sustained activation of JNK/p38 MAPK and apoptosis in a reactive oxygen species-dependent manner in neuroblastoma cells, Int. J. Cancer 112 (2004) 219–224.
[24] S. Tamura, H. Hosoi, Y. Kuwahara, K. Kikuchi, O. Otabe, M. Izumi, K. Tsuchiya, T. Iehara, T. Gotoh, T. Sugimoto, Induction of apoptosis by an inhibitor of EGFR in *neuroblastoma cells*, *Biochem. Biophys. Res. Commun.* 358 (2007) 226–232.
[25] H. Kuroda, T. Sugimoto, K. Ueda, S. Tsuchida, Y. Horii, J. Inazawa, K. Sato, T. Sawada, Different drug sensitivity in two neuroblastoma cell lines established from the same patient before and after chemotherapy, Int. J. Cancer 47 (1991) 732–737.
[26] H. Kuroda, T. Sugimoto, Y. Horii, T. Sawada, Signaling pathway of ciliary neurotrophic factor in human neuroblastoma cell lines, Med. Pediatr. Oncol. 36 (2001) 118–121.
[27] T. Sugimoto, H. Kuroda, Y. Horii, H. Moritake, T. Tanaka, S. Hattori S, Signal transduction pathways through TRK-A and TRK-B receptors in human neuroblastoma cells, Jpn. J. Cancer Res. 92 (2001) 152–160.
[28] F. Saito-Ohara, I. Imoto, J. Inoue, H. Hosoi, A. Nakagawara, T. Sugimoto, J. Inazawa, PPM1D is a potential target for 17q gain in neuroblastoma, Cancer Res. 63 (2003) 1876–1883.
[29] S.L. Cohn, A.D. Pearson, W.B. London, T. Monclair, P.F. Ambros, G.M. Brodeur, A. Faldum, B. Hero, T. Iehara, D. Machin, V. Mosseri, T. Simon, A. Garaventa, V. Castel, *K.K. Matthay, The International Neuroblastoma Risk Group (INRG) classification* system: an INRG Task Force report, J. Clin. Oncol. 27 (2009) 289–297.
[30] R.C. Seeger, G.M. Brodeur, H. Sather, A. Dalton, S.E. Siegel, K.Y. Wong, D. Hammond, Association of multiple copies of the *N-MYC* oncogene with rapid progression of neuroblastomas, N. Engl. J. Med. 313 (1985) 1111–1116.
[31] G.M. Brodeur, F.A. Hayes, A.A. Green, J.T. Casper, J. Wasson, S. Wallach, R.C. Seeger, Consistent *N-MYC* copy number in simultaneous or consecutive neuroblastoma samples from sixty individual patients, Cancer Res. 47 (1987) 4248–4253.

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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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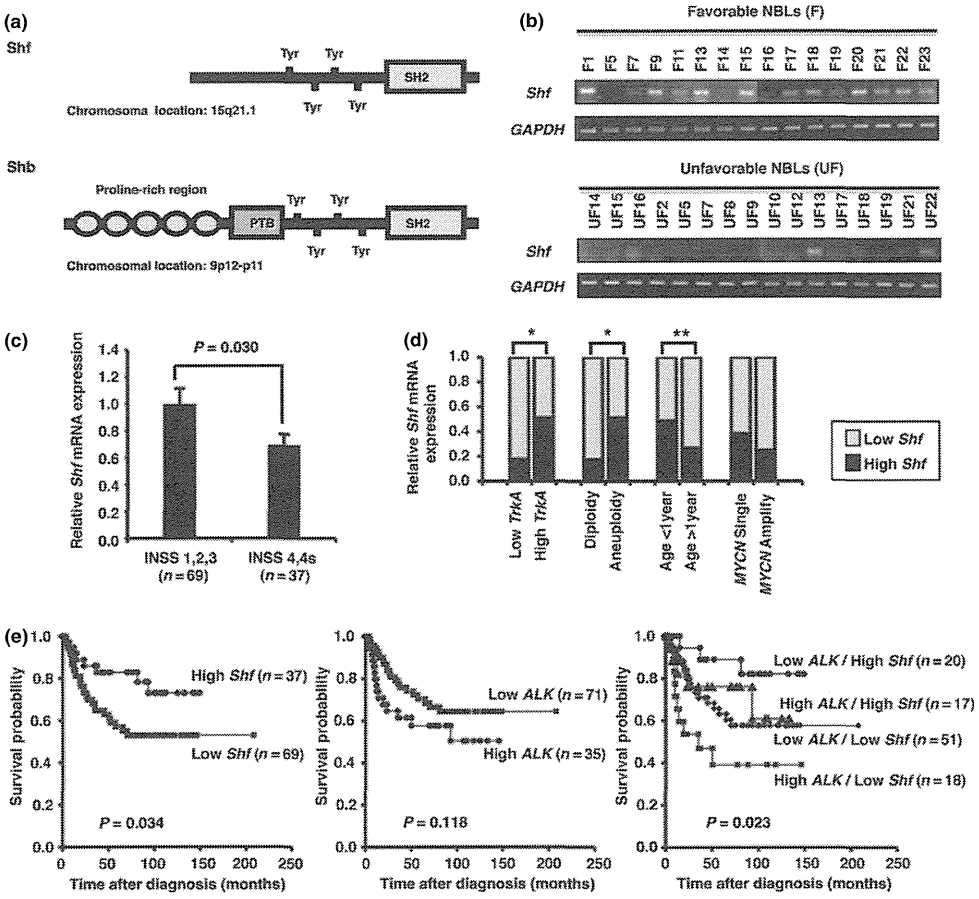

Fig. 1. Expression profiles of *Shf* mRNA in primary neuroblastoma (NBL). (a) Structural differences between Shf and Shb adaptor proteins. PTB, phosphotyrosine-binding domain; SH2, Src homology 2 domain; Tyr, tyrosine. (b) Differential expression of *Shf* in neuroblastomas with favorable (F) and unfavorable (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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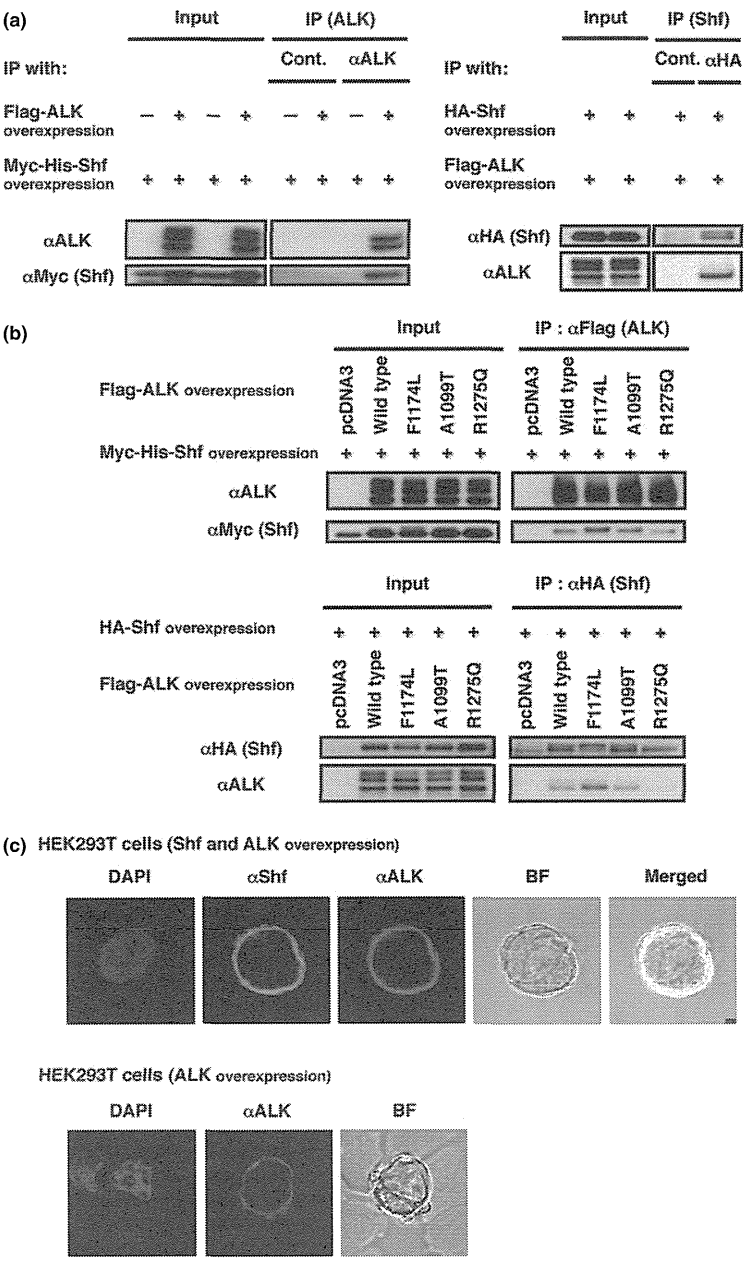

Fig. 2. Physical interaction between adaptor protein Shf and anaplastic lymphoma kinase (ALK). (a) Immunoprecipitation (IP) in 293T cells. Flag-tagged ALK and either HA-tagged or Myc-His-tagged Shf were exogenously overexpressed. Cont., control. (b) Immunoprecipitation assay under the exogenous expression 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

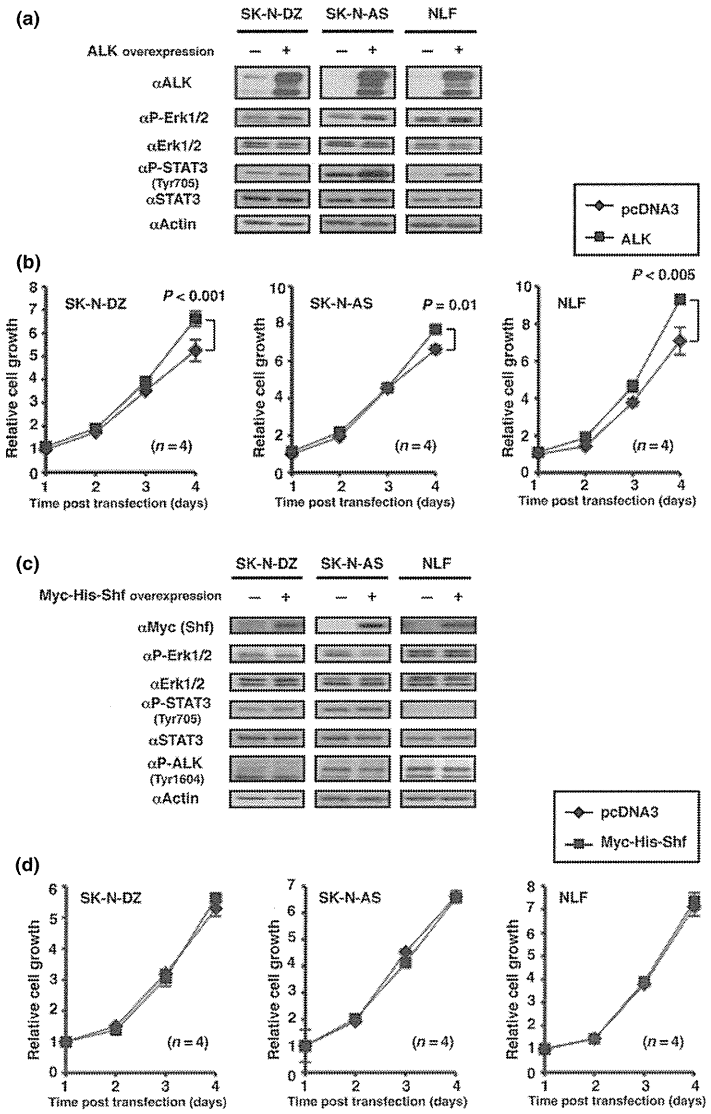

Fig. 3. Overexpression of anaplastic lymphoma kinase (ALK) facilitates cell growth and activates downstream signal pathways. (a) ALK overexpression induced phosphorylation of Erk1/2 and signal transducer and activator of transcription 3 (STAT3) in neuroblastoma cell lines SK-N-AS, SK-N-DZ, and NLF. (b) 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, phopho-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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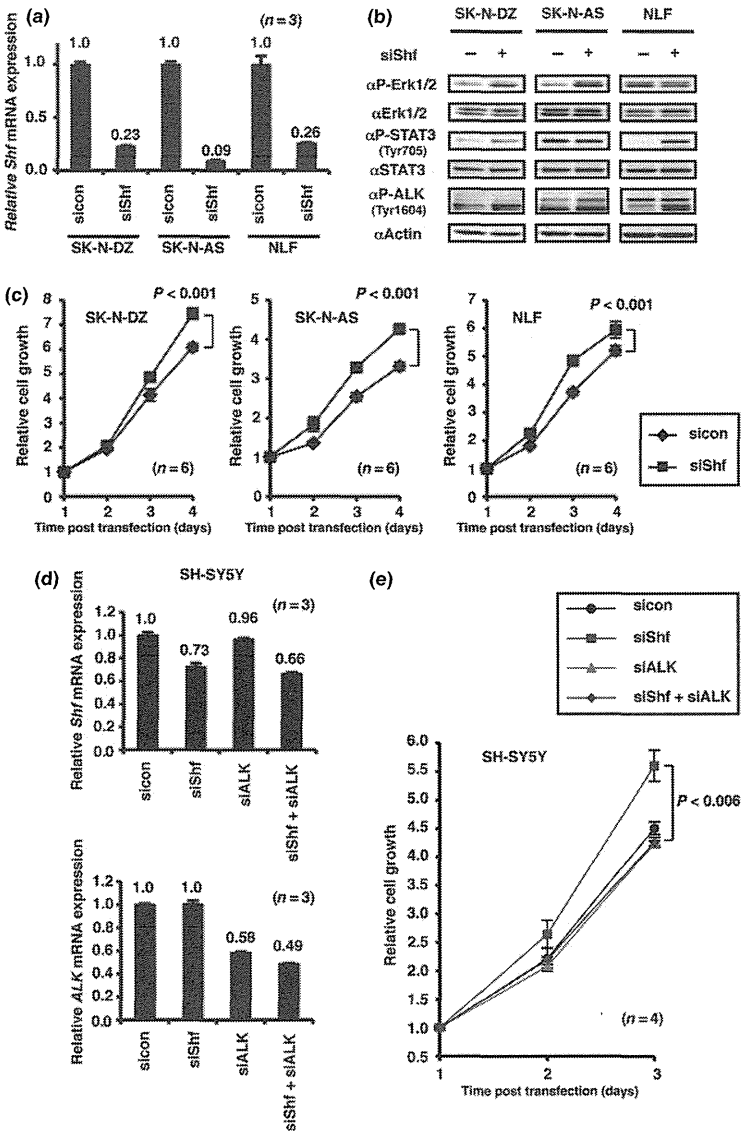

Fig. 4. Knockdown of *Shf* facilitates cell growth as well as activation of the anaplastic lymphoma kinase (ALK) pathway. (a) Knockdown of *Shf* mRNA mediated by specific siRNA was confirmed by real-time PCR. (b) *Shf* knockdown induced phosphorylation of ALK itself, Erk1/2, and signal transducer and 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

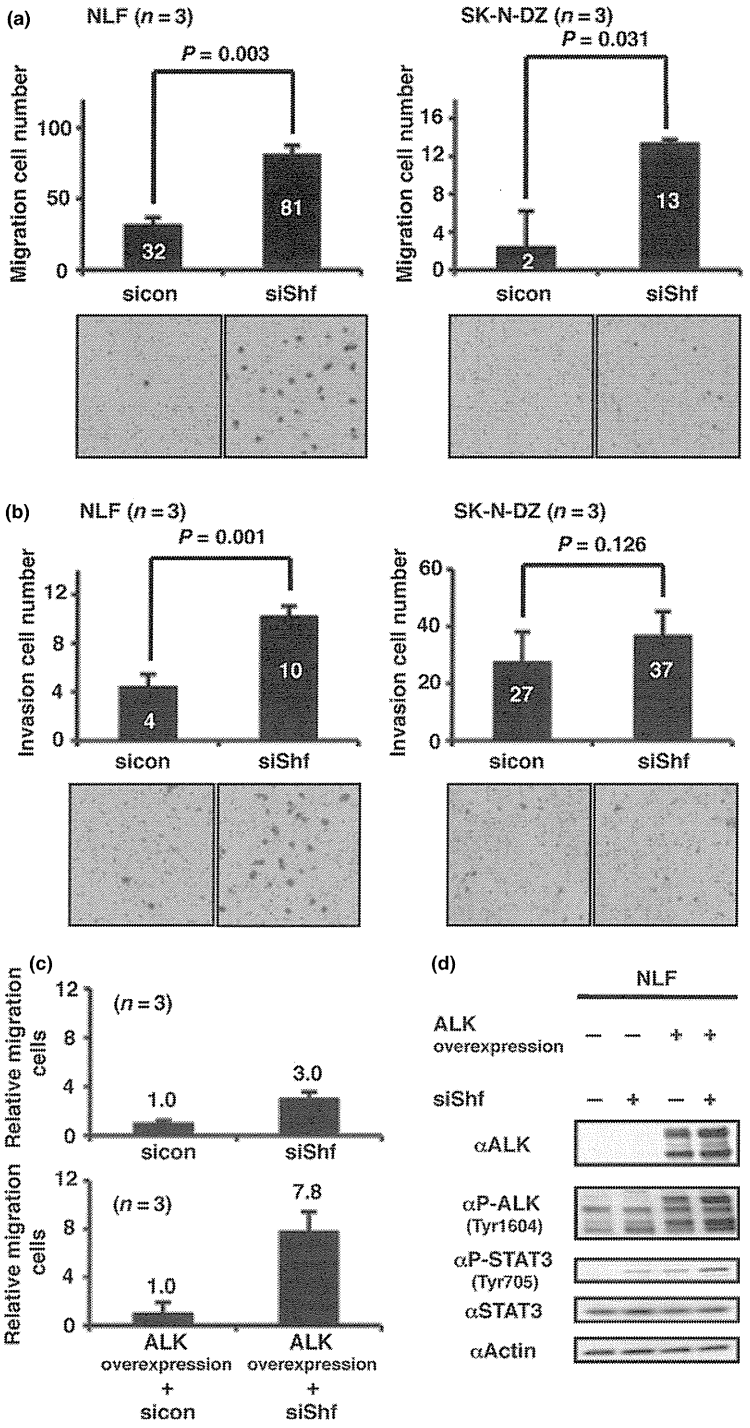

Fig. 5. Knockdown of *Shf* mediated by siRNA increases cellular motility and invasion capability in NLF and SK-N-DZ neuroblastoma cell lines. (a) Cellular migration was stimulated by knockdown of *Shf*. Mean values were calculated from independent triplicate experiments. Error bars indicate standard 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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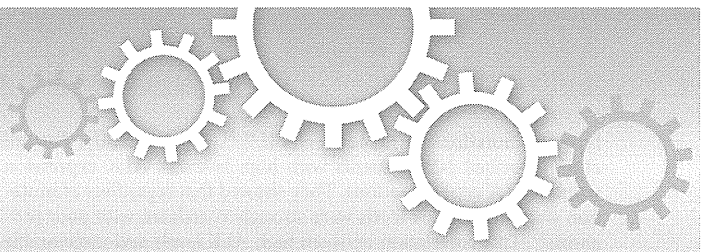

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ALK is a MYCN target gene and regulates cell migration and invasion in neuroblastoma

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Human anaplastic lymphoma kinase (*ALK*) has been identified as an oncogene that is mutated or amplified in NBLs. To obtain a better understanding of the molecular events associated with ALK in the pathogenesis of NBL, it is necessary to clarify how *ALK* gene contributes to NBL progression. In the present study, we found that *ALK* expression was significantly high in NBL clinical samples with amplified *MYCN* ($n = 126$, $P < 0.01$) and in developing tumors of *MYCN*-transgenic mice. Indeed, promoter analysis revealed that *ALK* is a direct transcriptional target of MYCN. Overexpression and knockdown of ALK demonstrated its function in cell proliferation, migration and invasion. Moreover, treatment with an ALK inhibitor, TAE-684, efficiently suppressed such biological effects in *MYCN* amplified cells and tumor growth of the xenograft in mice. Our present findings explore the fundamental understanding of ALK in order to develop novel therapeutic tools by targeting ALK for aggressive NBL treatment.

Neuroblastoma (NBL) is an embryonal malignancy derived from precursor cells of the sympathetic nervous system, and accounts for 7–10% of childhood cancers and around 15% of cancer deaths in children[1]. Though some subsets of NBL undergo spontaneous regression without therapy, about 60–70% of high-risk NBL patients are resistant to currently available therapies and have poor prognoses[1–3]. The genetic feature most consistently associated with treatment failure is an amplification of the *MYCN* proto-oncogene, which is strongly correlated with advanced disease[4–7]. Even in otherwise favorable localized disease, *MYCN* amplification indicates poor outcome, underscoring its biological importance. Indeed, upregulation of MYCN in NBL cells resulted in accelerated proliferation, migration and invasion[8–11]. Consistent with these observations, transgenic mice overexpressing MYCN in neural crest-derived tissues displayed frequent development of NBL[12], suggesting that upregulated expression of MYCN is causative in the genesis and development of NBL *in vivo*. However, the role of *MYCN* expression and its molecular mechanisms to induce an aggressive phenotype are still unclear. Identification of its direct transcriptional target gene(s) may provide a novel insight into understanding the functional contribution of MYCN in malignant phenotypes of aggressive NBL.

The *MYC* family of proto-oncogenes belongs to the basic helix-loop-helix leucine-zipper class of transcription factors. MYC proteins (MYCN and c-Myc) share several regions of homology and similar cellular functions that target proliferative pathways vital for cancer progression. Members of this family function as heterodimers with MAX, and exert transcriptional activity by specifically binding to a consensus E-box motif (CACGTG) located within the promoter regions of a diverse set of target genes[13–15]. Although a handful of MYCN target genes involved in MYCN-driven cell proliferation and apoptosis have been identified, the target genes responsible for MYCN-mediated cell migration and invasion remain elusive.

Anaplastic lymphoma kinase (*ALK*) has been identified as a gene upregulated in unfavorable NBL, suggesting a possible oncogenic role for this receptor tyrosine kinase, which was previously linked with NBL[16,17]. Recently, *ALK* point mutations were described in 3–11% of sporadic NBL, and were found to be one of the most important