

Tumorigenesis and Neoplastic Progression

Biological Role of Anaplastic Lymphoma Kinase in Neuroblastoma

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Anaplastic lymphoma kinase (ALK) is a tyrosine kinase receptor originally identified as part of the chimeric nucleophosmin-ALK protein in the t(2;5) chromosomal rearrangement associated with anaplastic large cell lymphoma. We recently demonstrated that the ALK kinase is constitutively activated by gene amplification at the ALK locus in several neuroblastoma cell lines. Forming a stable complex with hyperphosphorylated ShcC, activated ALK modifies the responsiveness of the mitogen-activated protein kinase pathway to growth factors. In the present study, the biological role of activated ALK was examined by suppressing the expression of ALK kinase in neuroblastoma cell lines using an RNA interference technique. The suppression of activated ALK in neuroblastoma cells by RNA interference significantly reduced the phosphorylation of ShcC, mitogen-activated protein kinases, and Akt, inducing rapid apoptosis in the cells. By immunohistochemical analysis, the cytoplasmic expression of ALK was detected in most of the samples of neuroblastoma tissues regardless of the stage of the tumor, whereas significant amplification of ALK was observed in only 1 of 85 cases of human neuroblastoma samples. These data demonstrate the limited frequency of ALK activation in the real progression of neuroblastoma. (*Am J Pathol* 2005, 167:213–222)

Receptor tyrosine kinases (RTKs) play an important role in regulating diverse cellular processes, such as prolifer-

ation, differentiation, survival, motility, and malignant transformation. The activation of RTKs typically requires ligand-induced receptor oligomerization, which results in tyrosine autophosphorylation of the receptors at tyrosine residues.^{1–3} By recruiting specific sets of signal transducer molecules in a phosphorylation-dependent manner, each RTK is capable of inducing individual, specific cellular responses.⁴ On the other hand, activation of RTKs by either mutations or overexpression is frequently found in various human malignancies.^{3,5}

Anaplastic lymphoma kinase (ALK) is a 200-kd tyrosine kinase encoded by the *ALK* gene on chromosome 2p23. ALK was first identified as part of an oncogenic fusion tyrosine kinase, nucleophosmin-ALK, which is associated with anaplastic large cell lymphoma.^{6,7} It was also found as a form of fusion protein with a clathrin heavy chain (CTCL) in myofibroblastic tumors.⁸ Full-length ALK has the typical structure of an RTK, with a large extracellular domain, a lipophilic transmembrane segment, and a cytoplasmic tyrosine kinase domain.^{9,10} ALK is highly homologous to leukocyte tyrosine kinase (LTK) and is further classified into the insulin receptor superfamily. The *LTK* gene is mainly expressed in pre-B lymphocytes and neuronal tissues,^{11–13} whereas expression of the normal *ALK* gene in hematopoietic tissues has not been detected. Instead, it is dominantly expressed in the neural system.^{14,15} In the developing brains of mice, specific expression of *ALK* was seen in the thalamus, mid-brain, olfactory bulb, and selected cranial regions, as well as the dorsal root, the ganglia of mice,^{9,10,16} suggesting a specific role in the development of the embryonic nervous system. Currently, however, the function of ALK in adult normal tissue or carcinogenesis remains an open question. Several studies have recently indicated pleiotrophin or midkine as possible ligands for ALK.^{17,18} Although they appeared to induce the functional activa-

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tion of ALK, it is still unclear whether these molecules are the physiological ligands of ALK.

Neuroblastoma is one of the most common pediatric tumors derived from the sympathoadrenal lineage of the neural crest. Tumors found in patients under the age of 1 year are usually favorable and often show spontaneous differentiation and regression.¹⁹ Amplification of the *N-myc* gene occurs in approximately 25% of neuroblastomas and correlates with the aggressiveness of the disease. In addition to *N-myc* gene amplification, the expression of various genes has significant correlation with the stage of and prognosis for neuroblastoma. A high level of TrkA expression is predictive of a favorable outcome,²⁰ whereas TrkB is highly expressed in immature neuroblastomas with *N-myc* amplification.²¹ High expression of caspase-1, -3, and -8 is correlated with favorable neuroblastomas.^{22,23} On the other hand, survivin, which suppresses caspase and promotes the cell survival signal, is significantly expressed,²⁴ and telomerase is activated²⁵ in unfavorable tumors. There may be a critical difference in the expression of other molecules, including RTKs, in neuroblastoma. A recent paper showed that full-length ALK is detected in almost one-half of the cell lines derived from neuroblastomas and neuroectodermal tumors.²⁶ We have recently shown using mass-spectrometry analysis that ALK is a major phosphoprotein associated with hyperphosphorylated ShcC in several neuroblastoma cell lines.²⁷ In these cells, ALK was markedly activated, and it induced the constitutive phosphorylation of ShcC and mitogen-activated protein kinase (MAPK), regardless of stimulation by epidermal growth factor (EGF) or nerve growth factor.²⁷ These findings strongly suggest that constitutively activated ALK kinase plays a physiological role in the development of neuroblastoma.

In this study, we investigated the biological function of the constitutively activated ALK kinase in neuroblastoma. The RNA interference (RNAi) technique using specific sets of small interfering RNA (siRNA) was induced to inhibit the *ALK* gene expression in human neuroblastoma cells with or without gene amplification of *ALK*. The effects of disrupted ALK expression on cell survival or downstream signaling, such as MAPKs or Akt pathways, are examined to understand the biological meaning of ALK amplification in neuroblastoma cells. We also performed Southern blot analysis of primary neuroblastoma tumors from 85 patients to check whether the *ALK* gene amplification was actually present in neuroblastoma tissues. Furthermore, we sought the *ALK* gene expression in human neuroblastoma tissues using immunohistochemical analysis.

Materials and Methods

Cell Culture

Cell lines of human neuroblastoma were maintained in RPMI 1640 supplemented with 10% fetal calf serum (Sigma, St. Louis, MO), penicillin, and streptomycin at 37°C in a humidified 5% CO₂ incubator.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was extracted with ISOGEN (Nippongene Japan, Toyama, Japan) from NB-39-nu and SK-N-MC cells. The PCR primer pair 5'-AGGTTCTGGCTGCAGATGGT-3' and 5'-ACATTGTTCTCTCGAGTGCAGAC-3' corresponding to the cytoplasmic portion of human ALK was prepared. As much as 0.25 µg of total RNA was reverse transcribed and amplified with the SuperScript One-step RT-PCR with the Platinum Taq kit (Invitrogen Life Technologies, Carlsbad, CA) in a total volume of 50 µl including 2× reaction mix, 0.2 µmol/L of each primer, and 1 µl of RT/Platinum Taq Mix. Amplification conditions consisted of cDNA synthesis and predenaturation at 50°C for 30 minutes and 94°C for 2 minutes followed by 25 cycles at 94°C for 15 seconds, 58°C for 30 seconds, and 72°C for 45 seconds. A final amplification for 7 minutes at 72°C finished the PCR. The product was separated with 1.2% agarose gel electrophoresis and analyzed using the Quality One System (Bio-Rad, Hercules, CA).

Immunochemical Analysis of Proteins

Immunoprecipitation and immunoblotting were performed as described previously.²⁷ The polyclonal antibodies against the CH1 domains of ShcC (amino acids 306–371) and the anti-ALK antibody (αALK) that was against the cytoplasmic portion (amino acid 1379–1524) of human ALK were prepared as described previously.^{27,28} An anti-phosphotyrosine antibody (4G10) was obtained from UBI. Anti-p44/42 MAPKs, anti-phospho-p44/42 MAPKs, anti-Akt, and anti-phospho-Akt antibodies were purchased from Cell Signaling (Beverly, MA). Anti-EGF receptor (EGFR), anti-Ret, and anti-TrkA antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). *In vitro* kinase assay for ALK was performed as previously described.²⁷ Anti-ALK immunoprecipitates were incubated with or without Poly-Glu/Tyr as an exogenous substrate.

Immunocytochemistry

For ALK/TOTO-3, immunostaining using anti-ALK antibody was performed at first, and then nuclei were stained using TOTO-3. The cells seeded on the 24-well plates were washed with phosphate-buffered saline (PBS) three times and fixed with 4% paraformaldehyde (methanol free) for 5 minutes at room temperature. The cells were rinsed with PBS twice and then permeabilized with 0.2% Triton X-100 solution in PBS for 10 minutes at room temperature. The cells were blocked with 5% goat serum and 3% bovine serum albumin-Tris-buffered saline for 30 minutes at room temperature. The blocking solution was drained off, and the cells were incubated with a 1:1000 dilution of αALK for 1 hour at room temperature. The cells were rinsed with PBS three times and incubated with a 1:2000 dilution of Alexa fluor (Molecular Probes, Eugene, OR) and 1:100 dilution of TOTO-3 (Molecular Probes) for

Table 1. Patient Characteristics of Neuroblastoma Tissues with *ALK* Gene Gain or Amplification

Case	Age*	Primary tumor		Copy nos. of <i>ALK</i> [‡]	Amplification of <i>N-myc</i> (n)
		Location	Clinical stage [†]		
1	3y5m	Adrenal gland	IV	2.0 ± 0.2	+ (35)
2	5y0m	Peritoneum	IV	1.8 ± 0.1	+ (>150)
3	2y7m	Abdomen	IV	2.1 ± 0.8	+ (150)
4	8m	Adrenal gland	I	3.0 ± 1.0	—
5	4y9m	Abdomen	IV	2.0 ± 0.2	—
6	3y9m	Adrenal gland	III	2.7 ± 0.2	+ (>150)
7	1y4m	Adrenal gland	IV	2.8 ± 1.0	+ (150)
8	1y7m	Adrenal gland	IV	9.5 ± 2.2	+ (>100)

*Age of onset: year (y), month (m).

[†]The staging criterion was based on the International Neuroblastoma Staging System.

[‡]The averages of the calculated copy numbers from three independent blottings are shown.

30 minutes at room temperature. The cells were washed three times with PBS and mounted in glycerol-based 2.5% 1,4-diazabicyclo[2,2,2] octan. Confocal laser scanning analysis was carried out. For *ALK*/TUNEL, we first carried out TUNEL and then proceeded to standard immunocytochemistry using anti-*ALK* antibody. TUNEL was performed using the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI) with the following modifications. The NB-39-nu cells seeded on the 24-well plates that were treated with siRNAs were washed with PBS twice and fixed with 4% paraformaldehyde (methanol free) for 25 minutes at 4°C. The cells were rinsed with PBS twice and then permeabilized with 0.2% Triton X-100 solution in PBS for 5 minutes at room temperature. The cells were washed with PBS twice and covered with an equilibration buffer (from the kit) for 10 minutes at room temperature. The equilibration buffer was drained off, and a reaction buffer containing the equilibration buffer, nucleotide mix, and terminal deoxynucleotidyl transferase enzyme was added to the cells and incubated at 37°C for 1 hour, avoiding exposure to light. The cells were incubated for 15 minutes at room temperature with 2× standard saline citrate to stop the reaction. The cells were washed with PBS three times and then stained for *ALK* using immunofluorescence as follows. The cells were blocked with 2% bovine serum albumin (Boehringer Mannheim, Germany) for 30 minutes at room temperature. The blocking solution was drained off, and the cells were incubated with a 1:1000 dilution of α*ALK* for 1 hour at room temperature. The cells were rinsed with PBS three times and incubated with a 1:40 dilution of rhodamine-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology) for 30 minutes at room temperature. The cells were washed three times with PBS and then mounted and observed in the same manner as that for *ALK*/TOTO-3.

DNA Extraction and Southern Blotting

Genomic DNAs derived from neuroblastoma cell lines were obtained from cultured cells as described using the procedure of Perucho et al.²⁹ Samples of 85 neuroblastoma tissues were collected at the Chiba Cancer Center and stored as forms of genomic DNA. The characteristics of some of these patients are shown in Table 1. The stage

criterion was based on the International Neuroblastoma Staging System.³⁰ Samples of 5 μg of DNA digested by *Eco*RI were electrophoresed in 0.8% agarose gel and blotted onto nitrocellulose filters (Hybond-N+; Amersham, Piscataway, NJ). The probes for detecting the *ALK* gene, *N-myc* gene, and *ShcC* gene were used in our previous study.²⁷ The intensities of these signals were measured using a Molecular Imager FxPro (Bio-Rad). This study was approved by the ethical judging committee of the National Cancer Center and the Chiba Cancer Center of Japan.

RNA Interference Technique

Twenty-one-nucleotide double-stranded RNAs were synthesized and purified using Dharmacon Research (Lafayette, CO). To suppress the expression of *ALK* protein, two different pairs of *ALK* siRNAs, *ALK*-siRNA1 and *ALK*-siRNA2, were obtained. The sequences were 5'-GAGUCUGGCAGUUGACUUCdTdT-3' for *ALK*-siRNA1 and 5'-GCUCCGGCGUGCCAAGCAGdTdT-3' for *ALK*-siRNA2, corresponding to coding region 153 to 171 and 399 to 417 relative to the first nucleotide of the start codon, respectively. Entire sequences were derived from the sequence of human *ALK* mRNA (accession no. HSU62540). An siRNA, targeting a sequence in the firefly (*Photinus pyralis*) luciferase mRNA, was used as a negative control (Dharmacon) (*luc*-siRNA). We also used a scramble siRNA, Scramble Duplex II (Dharmacon) (*s*-siRNA) as a mismatch siRNA control in addition to *luc*-siRNA.

NB-39-nu cells were trypsinized, diluted with growth medium containing 10% fetal calf serum, and transferred to 12-well plates at 6 × 10⁴ cells per well for 24 hours before transfection. The transfection of siRNA was carried out using jetSI (Poly plus transfection). A total of 100 μl of serum-free growth medium and 4 μl of jetSI per well were preincubated for 5 to 10 minutes at room temperature. While the incubation was being performed, 100 μl of serum-free growth medium was mixed with 5 μl of 20 μmol/L siRNA duplex (100 pmol). Total siRNA amounts of 50, 100, and 200 pmol were checked in preliminary experiments to find out 100 pmol is the minimal and optimal amount in this scale of RNAi. The 100 μl of jetSI serum-free medium solution was added to the 100 μl of siRNA

duplex solution, gently mixed, and incubated for 30 minutes at room temperature. The growth medium on the cells was removed, and 800 μ l of serum-free medium was added to each well. A total of 200 μ l of the entire mixture was overlaid onto the cells, and cells were incubated for 4 hours at 37°C in a 5% CO₂ incubator. After incubation, 1 ml of medium containing 4% fetal calf serum was added without removing the transfection mixture (final concentration 2%). The cells were assayed 84 hours after transfection. SK-N-MC cells were seeded in 12-well plates at a concentration of 1.3×10^5 cells per well. These were treated with siRNAs in the same way as NB-39-nu and assayed 48 hours after transfection. In the 24-well plate, the cells were seeded at the same concentration as the 12-well plate, and siRNAs and all other reagents were used at half volume. After transfection, the cells were examined under a light microscope every day.

Double Staining for ALK and TUNEL

For double staining, we first carried out TUNEL and then proceeded to standard immunocytochemistry using anti-ALK antibody. TUNEL was performed using the DeadEnd Fluorometric TUNEL System (Promega) with the following modifications. The NB-39-nu cells seeded on the 24-well plates that were treated with siRNAs were washed with PBS twice and fixed with 4% paraformaldehyde (methanol free) for 25 minutes at 4°C. The cells were rinsed with PBS twice and then permeabilized with 0.2% Triton X-100 solution in PBS for 5 minutes at room temperature. The cells were washed with PBS twice and covered with an equilibration buffer (from the kit) for 10 minutes at room temperature. The equilibration buffer was drained off, and a reaction buffer containing the equilibration buffer, nucleotide mix, and terminal deoxynucleotidyl transferase enzyme was added to the cells and incubated at 37°C for 1 hour, avoiding exposure to light. The cells were incubated for 15 minutes at room temperature with 2 \times standard saline citrate to stop the reaction. The cells were washed with PBS three times and then stained for ALK using immunofluorescence as follows. The cells were blocked with 2% bovine serum albumin (Boehringer Mannheim) for 30 minutes at room temperature. The blocking solution was drained off, and the cells were incubated with a 1:1000 dilution of α ALK for 1 hour at room temperature. The cells were rinsed with PBS three times and incubated with a 1:40 dilution of rhodamine-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology) for 30 minutes at room temperature. The cells were washed three times with PBS and mounted in glycerol-based 2.5% 1,4-diazabicyclo[2,2,2] octan. Confocal laser scanning analysis was carried out.

DNA Fragmentation Assay

To detect apoptotic DNA cleavage, DNA fragmentation assay was performed using an Apoptotic DNA Ladder kit (Chemicon International, Inc., Temecula, CA). The cells seeded on the 12-well plates that were treated with siRNAs as previously mentioned were collected in 1.5-ml

microcentrifuge tubes. The cells were washed with PBS, centrifuged, and lysed with 20 μ l of TE lysis buffer. The lysates were incubated with 5 μ l of enzyme A (RNase A) at 37°C for 10 minutes and then at 55°C for 30 minutes after the addition of 5 μ l of Enzyme B (Proteinase K). Afterward, 5 μ l of ammonium acetate solution and 100 μ l of absolute ethanol were added, and the samples were kept at -20°C for 10 minutes. The samples were centrifuged, and the pellets were washed with 70% ethanol. Then the DNA pellets were dissolved in 30 μ l of DNA suspension buffer. DNA fragmentations were visualized by electrophoresis on 2% agarose gel containing ethidium bromide.

Immunohistochemistry

As for positive control, tumor xenograft was made by injection of NB-39-nu cells subcutaneously in 5-week-old SCID mice. Immunohistochemical staining with ALK antibody (α ALK) (1:1000), was performed on 16 human neuroblastoma tumors selected from the surgical pathology file at the Department of Pathology, Aichi Medical University based on the results of histopathology evaluation³¹ and *N-myc* status. All of those tumor samples were obtained before chemotherapy and irradiation therapy and included nine favorable histology cases with nonamplified *N-myc* (FH&NA), two unfavorable histology cases with amplified *N-myc* (UH&A), and five unfavorable histology cases with nonamplified *N-myc* (UH&NA).

Four-micrometer-thick sections from the formalin-fixed and paraffin-embedded tissue samples were deparaffinized and microwaved for three times for 5 minutes in Na-citrate buffer (pH 6.0) for antigen retrieval. The slides were first immersed in 0.3% hydrogen peroxide in methanol for 20 minutes and then in 10% normal goat serum for 30 minutes. The primary antibody (α ALK) was then applied at 4°C overnight, followed by a standard staining procedure using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Sections were counterstained with hematoxylin for light microscopic review and evaluation. ALK was always positively detected in the cytoplasm of NB-39-nu tumor xenograft and in the cytoplasm and neuritic processes of normal ganglion cells in the separate positive control sections as well as in the test sections as built-in control, whenever available. As for the negative controls, normal rabbit immunoglobulins (1:500 dilution; Vector Laboratories) or preimmune serum for α ALK (1:1000 dilution) was applied as the primary antibody.

Results

Significant Amplification of the ALK Gene and Constitutive Activation of ALK Kinase in Three Neuroblastoma Cell Lines

As shown in Figure 1A, NB-39-nu, Nagai, and NB-1 cells have significant levels of amplification of the *ALK* gene (30–40 copies per cell) among 25 neuroblastoma and neuroepithelioma cell lines examined. Other cell lines such as SK-N-MC have only one copy of the *ALK* gene just like the

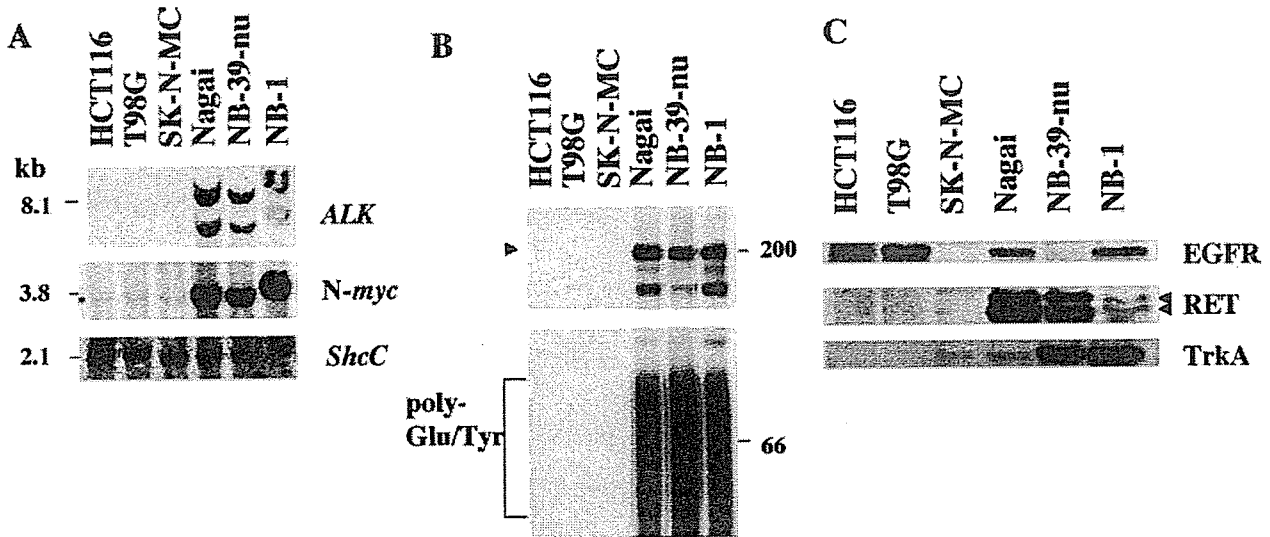


Figure 1. Marked gene amplification of the *ALK* locus and significant elevation of kinase activity of ALK in NB-39-nu, Nagai, and NB-1 cells. **A:** To detect *ALK* gene amplification, samples of 10 μ g of DNA were digested with *EcoRI*. Fragments of about 2.5, 3.1, 6.1, and 8.1 kb were detected using the 32 P-labeled probe prepared as previously described.²⁷ Amplification of the *N-myc* gene was detected using the same filter re-hybridized with the probe for *N-myc*. As a control for the amounts of DNA, the same filter was re-hybridized with the probe for *ShcC*. **B:** *In vitro* kinase assay of ALK in neuroblastoma cells immunoprecipitated with α ALK was performed as previously described.²⁷ Kinase reaction was performed without (**top panel**) or with (**bottom panel**) poly-Glu/Tyr (4:1) as exogenous substrates. Autophosphorylated ALK protein is marked by an arrow. Phosphorylated poly-Glu/Tyr is detected as smear indicated by the bracket. **C:** The expression patterns of other receptor tyrosine kinases in neuroblastoma cell lines. Each cell line was harvested, and about 30 μ g of whole-cell lysates was subjected to Western blot analysis using the antibodies as indicated on the right. RET proteins are marked by arrows.

other types of solid tumor cell lines used as controls. *In vitro* kinase assay revealed outstanding ALK kinase activity in these three cell lines compared with other cells (Figure 1B), which is consistent with our previous study.²⁷ To examine whether overexpressed and activated ALK affects the expression of other RTKs in these cells, protein expression levels of RTKs, including EGFR, Ret, and TrkA, are compared with other cell lines. Significantly high levels of expression of EGFR and TrkA were observed in two of three cell lines overexpressing ALK (Figure 1C, top and bottom). Ret expression was commonly elevated in all three cell lines with activated ALK, especially in Nagai and NB-39-nu (Figure 1C, middle), consistent with previous study by Northern blotting.³² Although it is unknown whether overexpression of these RTKs is related to overexpression of ALK, no obvious down-regulation of other RTKs was found in these ALK-amplified cell lines.

Inhibition of Activated ShcC, MAPKs, and Akt by Suppressing Activated ALK

To investigate the effect of suppressing the ALK expression level in ALK-amplified neuroblastoma cells using the RNAi technique, we synthesized two different RNA duplexes directed against nucleotide positions 153 to 171 and 399 to 417 within coding region *ALK* cDNA (ALK-siRNA1 and ALK-siRNA2, respectively). Because co-transfection of ALK-siRNA1 and ALK-siRNA2 was very effective in suppressing ALK expression, we performed all experiments presented here using a combination of two siRNAs, although similar results were obtained using only ALK-siRNA2. A sequence against the firefly luciferase gene (*luc*-siRNA) was used as a negative control. The expression of ALK protein is remarkably elevated in

NB-39-nu and Nagai compared with other neuroblastoma cell lines, such as SK-M-MC (Figure 2A), caused by gene amplification.²⁷ The RNA duplexes were transfected into NB-39-nu cells with *ALK* gene amplification and SK-N-MC cells containing only a single copy of the *ALK* gene. We also tried to introduce ALK-siRNAs in several different neuroblastoma cell lines with or without *ALK* amplification in addition to NB-39-nu and SK-N-MC cells, resulting in partial or no reduction of ALK expression presumably due to the unsuccessful introduction in those cells. Therefore, we decided to use these two cell lines to perform further analysis of the effect of ALK knockdown by RNAi technique. RT-PCR analysis revealed that ALK mRNA level was reduced in both NB-39-nu cells and SK-N-MC cells treated with ALK-siRNAs, not in the cells treated with *luc*-siRNA and *s*-siRNA (Figure 2B). Both expression and phosphorylation of ALK kinase were significantly suppressed in the NB-39-nu cells treated with ALK-siRNAs compared with a mock-transfection control or cells treated with *luc*-siRNA (Figure 2C). In these cells, phosphorylation of ShcC was also suppressed despite the unchanged total amount of ShcC (Figure 2C), demonstrating that ShcC is a potent substrate of activated ALK kinase and that activation of ALK is actually responsible for the hyperphosphorylation of ShcC in these cancer cells. While the expression of downstream molecules, such as p44/42 MAPKs and Akt, was not affected by ALK-siRNAs, phosphorylation of these molecules was markedly reduced (Figure 2C). These results suggest that the Ras-MAPK pathway and the phosphatidylinositol 3-kinase/Akt pathway are dominantly regulated by activated ALK kinase in these cells. Interestingly, in SK-N-MC cells treated with ALK-siRNAs, phosphorylation levels of ShcC, p44/42 MAPKs, and Akt were not affected by

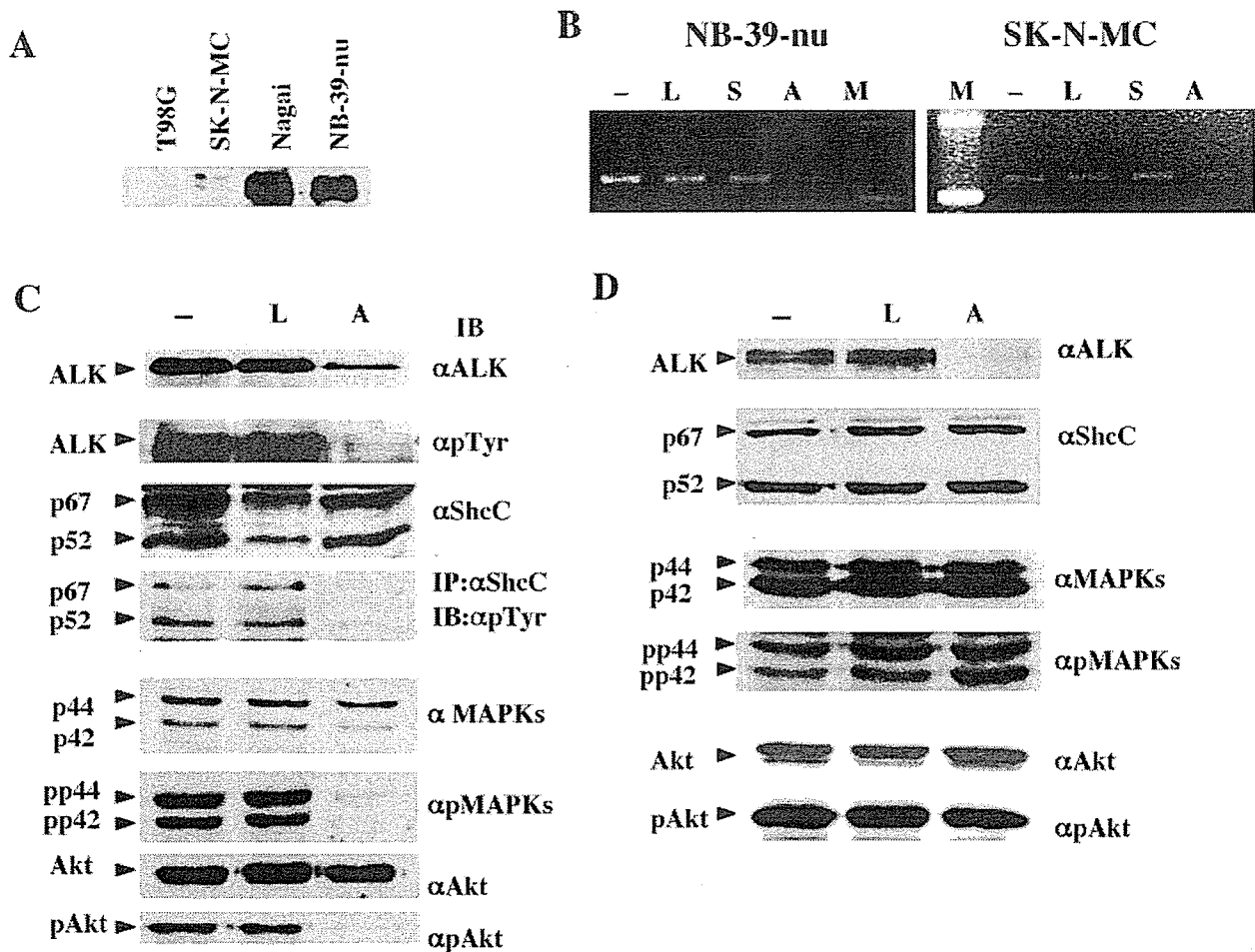


Figure 2. Suppression of ALK expression by siRNAs and changes in downstream molecules NB-39-nu cells and SK-N-MC cells. **A:** Expression levels of ALK protein in neuroblastoma cell lines including NB-39-nu and SK-N-MC. Each cell line was harvested, and about 30 μ g of whole-cell lysates was subjected to Western blot analysis using α ALK. **B:** mRNA levels of *Alk* in NB-39-nu cells. The cells were lysed at 84 hours after transfection and analyzed by RT-PCR. -, mock transfection; L, cells treated with luc-siRNA; S, cells treated with s-siRNA; A, cells treated with ALK-siRNAs; M, marker. **C:** NB-39-nu cells were harvested 84 hours after transfection. About 10 μ g of whole-cell lysates or 250 μ g of lysates immunoprecipitated with α ShcC was subjected to Western blot analysis using the antibodies as indicated on the right. -, mock transfection; L, cells treated with luc-siRNA; A, cells treated with ALK-siRNAs. **D:** SK-N-MC cells were harvested 48 hours after transfection. About 10 μ g of whole-cell lysates was subjected to Western blot analysis using the antibodies as indicated on the right. Bands of ShcC are marked by **arrows**. -, mock transfection; L, cells treated with luc-siRNA; A, cells treated with ALK-siRNAs.

ALK-siRNAs despite further suppression of the basal ALK expression level (Figure 2D), indicating that these pathways are not under the control of ALK in SK-N-MC cells.

Induction of Apoptosis by Suppression of Activated ALK

At 84 hours after transfection, apoptotic morphological changes, such as cell rounding, cytoplasmic blebbing, and irregularities of shape, were observed in NB-39-nu cells treated with ALK-siRNAs, whereas no significant changes were seen in the mock-transfected cells or in the luc-siRNA and the s-siRNA treated cells (Figure 3A). These morphological changes were not observed in SK-N-MC cells treated with ALK-siRNAs (data not shown). At 90 hours after transfection, NB-39-nu cells treated with ALK-siRNAs started to detach from the dish due to cell death.

To examine the localization of expression of ALK kinase, we performed double staining by anti-ALK anti-

body and TOTO-3, which stains the nucleus, in several neuroblastic cell lines. As shown in Figure 1D, unexpectedly, ALK protein overexpressed in NB-39-nu cells is localized in both membrane and cytoplasm. ALK staining was very weak in cell lines such as YT-nu and SK-N-MC with one copy of the *ALK* gene, however, its localization appeared to be the same as in NB-39-nu (data not shown). It was observed that the expression of ALK was completely lost after the RNAi-induced suppression of ALK (Figure 3C, top). To confirm whether the cell death resulted from apoptosis, cells were also analyzed by immunofluorescent TUNEL staining in NB-39-nu cells. TUNEL staining was clearly positive in these cells at 84 hours after transfection (Figure 3C, middle), indicating that apoptosis was induced in NB-39-nu cells treated with ALK-siRNAs. No significant TUNEL staining was observed in the mock-transfected cells or the luc-siRNA treated cells. Finally, DNA fragmentation assay was performed to measure the endonuclease activity accompa-

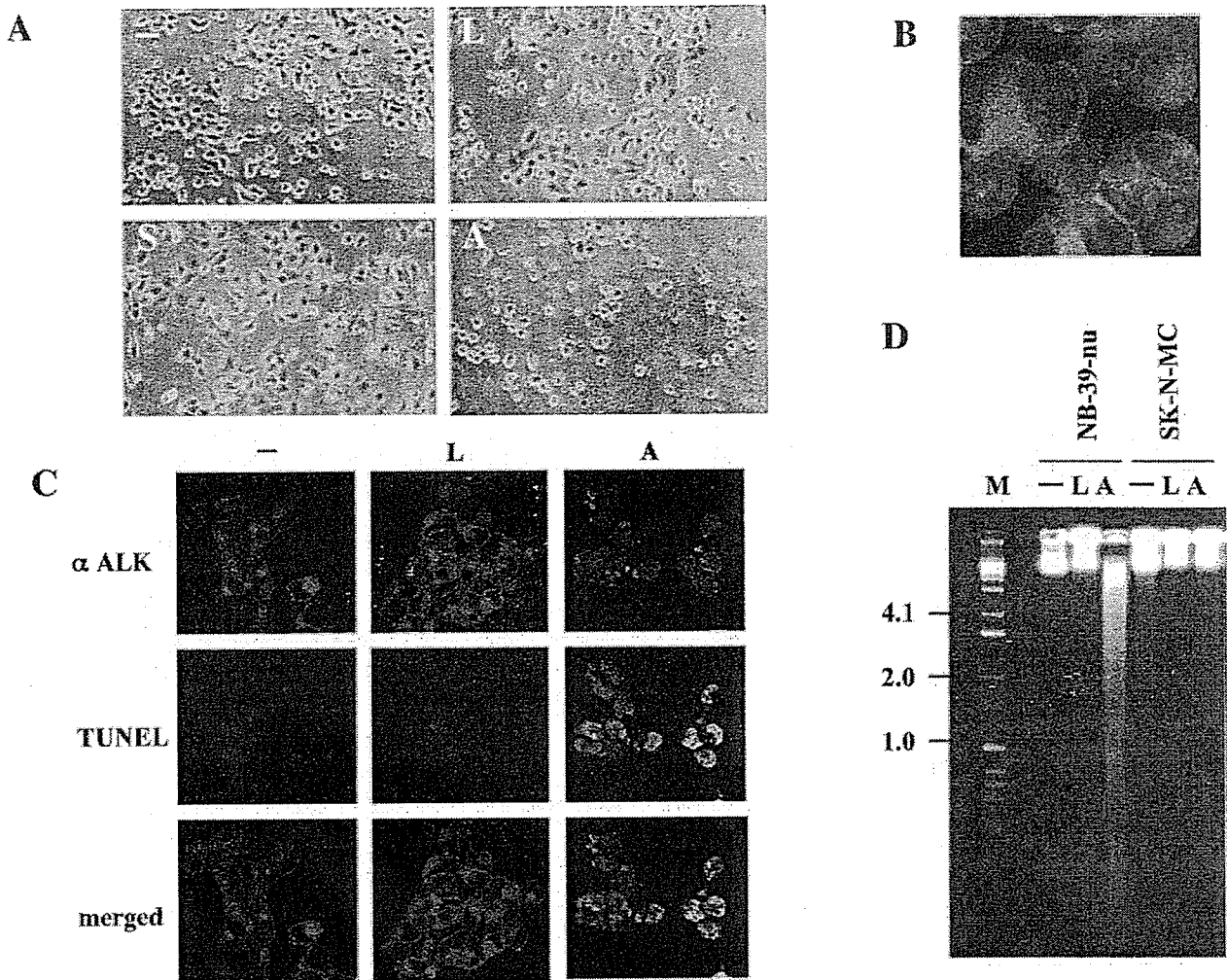


Figure 3. Induction of apoptosis in NB-39-nu cells treated with ALK-siRNAs. **A:** NB-39-nu cells on the dish were observed 84 hours after transfection under a light microscope. —, mock transfection; L, cells treated with luc-siRNA; S, cells treated with s-siRNA; A, cells treated with ALK-siRNAs. **B:** Cytoplasmic expression of ALK by immunocytochemistry. The cells were stained for the expression of ALK (red) and apoptotic cells by TOTO-3 (blue). **C:** Cells on 24-well plates were fixed, and TUNEL assay was followed by staining with α ALK (GST). The cells were stained for the expression of ALK (red) and apoptotic cells by TUNEL (green). —, mock transfection; L, cells treated with luc-siRNA; A, cells treated with ALK-siRNAs. **D:** DNA fragmentation assay in NB-39-nu cells and SK-N-MC cells treated with siRNAs. Genomic DNA was extracted 84 hours and 48 hours after transfection in NB-39-nu and in SK-N-MC, respectively. They were analyzed using electrophoresis. —, mock transfection; L, cells treated with luc-siRNA; A, cells treated with ALK-siRNAs; M, marker.

nied by apoptosis. The formation of significant DNA fragmentation was observed in the NB-39-nu cells but not in SK-N-MC cells treated with ALK-siRNAs (Figure 3D), indicating that cell apoptosis was induced through the suppression of ALK only in the NB-39-nu cells. This suggests that signaling pathways downstream of activated ALK dominantly regulate the survival of neuroblastoma cells with amplified ALK; therefore, the loss of ALK protein results in apoptotic changes to these cells.

Expression of ALK in Primary Neuroblastoma Tissues

Immunohistochemically, ALK was positively detected both in the cytoplasm of the neuroblastic cells and in the fine meshwork of neuropil of seven of nine tumors with favorable histology cases with nonamplified *N-myc* (FH&NA) (Figure 4, B and C). All seven unfavorable histology tumors (two

UH&A tumors and five UH&NA tumors) were positive in the cytoplasm and/or in the fine meshwork of neuropil for ALK (Figure 4A). There was no correlation between the frequency or intensity of ALK-staining and histology of neuroblastoma tissues, showing majority of neuroblastoma samples showed a detectable amount of ALK. There was no significant staining using preimmune serum from the same rabbit as that for anti-ALK antibody (data not shown). Essentially the same results were obtained using a mouse monoclonal antibody against human ALK (ALK1: DAKO) (data not shown).

Amplification of the ALK Gene in Primary Neuroblastoma Tissues

It is essential to show whether ALK overexpression or gene amplification occurs in actual human neuroblastoma tissues in addition to neuroblastoma cell lines.

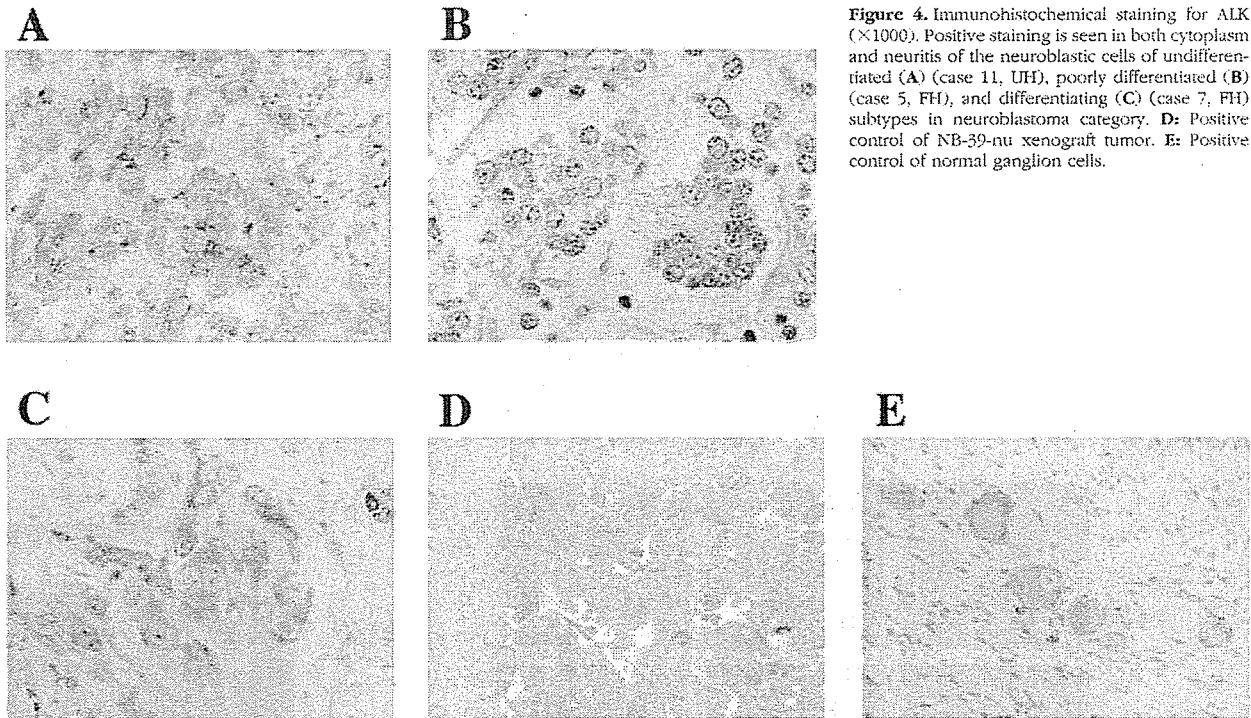


Figure 4. Immunohistochemical staining for ALK ($\times 1000$). Positive staining is seen in both cytoplasm and neuritis of the neuroblastic cells of undifferentiated (A) (case 11, UH), poorly differentiated (B) (case 5, FH), and differentiating (C) (case 7, FH) subtypes in neuroblastoma category. D: Positive control of NB-39-nu xenograft tumor. E: Positive control of normal ganglion cells.

Therefore, the mRNA amount of ALK kinase was first examined by RT-PCR on 32 primary neuroblastoma tissues (16 tissues with *N-myc* amplification and 16 tissues without *N-myc* amplification). Two of 32 cases showed slight elevation of *ALK* mRNA expression using several primer sets beyond the average expression level (data not shown).

To obtain more precise information about the copy numbers of *ALK*, we next analyzed the genomic DNAs of primary neuroblastoma tissues using Southern blot analysis. Whole purified DNA samples of tumors from 85 patients were examined. About the same number of *N-myc*-positive and *N-myc*-negative samples were collected to examine the relation between *Alk* and *N-myc* amplification. The intensities of signals on Southern blot membranes corresponding to the *ALK* gene and control *ShcC* gene, which is located on 9q22, were measured using a Molecular Imager FxPro (Bio-Rad), and the ratio of *ALK* signals to *ShcC* signals was calculated for each sample. Because more than 80% (70 samples) showed consistent ratios with each other in each experiment, these samples are treated as putative "single copy" controls. As several other samples showed apparently elevated intensity ratios, suggesting *ALK* amplification, relative copy numbers of *ALK* were calculated in comparison with average intensity ratios of putative single copy controls in each experiment. The results showed that there was significant *ALK* gene amplification in 8 of 85 patients (9.4%) (Figure 5). Seven of these eight cases, however, had only 1.8 to 3.0 copies of the *ALK* gene, suggesting a moderate gain of chromosomal focus rather than severe amplification. There was only one case that had outstanding amplification of *ALK* with approximately 10 copies. *N-myc* gene amplification was also detected

in this case. The characteristics of the eight patients with *ALK* gain or amplification are shown in Table 1. Whereas seven of eight patients were classified as Stage III or IV (one as Stage III and six as Stage IV), the rest was classified as Stage I. The case with *ALK* amplification had *N-myc* amplification and was classified as Stage IV. Seven of eight patients were more than 1 year of age.

Discussion

Studies on ALK kinase demonstrate that activated ALK is involved in malignant tumor formation as forms of fusion proteins that force oligomerization of this kinase. We recently showed that the intact form of ALK protein is con-

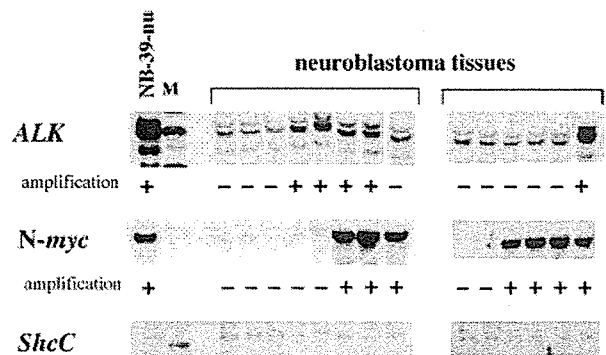


Figure 5. Detection of gene amplification of *ALK* and *N-myc* in primary neuroblastoma tissues. *ALK* was amplified in eight cases, and five of these eight cases are shown. The probe for *ALK* was removed from the filters, and the filters were re-hybridized in turn with other probes. Of eight cases with *ALK* amplification, *N-myc* amplification was detected in six cases and not detected in two cases. The probe for *ShcC* was used as a control for the amounts of DNA. M, marker.

stitutively activated by *ALK* gene amplification in three neuroblastoma cell lines, indicating a novel mechanism of activation of ALK kinase in malignancies.²⁷ In this study, amplification of the *ALK* gene was detected in primary neuroblastoma tissues for the first time. This suggests that activated ALK kinase plays a real role in the pathophysiology of neuroblastoma, such as giving a more malignant phenotype to the tumors by perturbing signal transduction. Recently, Motegi et al³³ showed that ALK transmits both mitogenic and differentiation signals, and that the MAPK pathway plays an important role in these effects in SK-N-SH cells without *ALK* gene amplification. Together with the fact that activated ALK surpasses regulation by other RTKs in cell lines with *ALK* gene amplification,²⁷ our new results showing apoptotic changes caused by the suppression of activated ALK protein clearly demonstrate the dominant role of ALK kinase in the survival of the *ALK*-amplified type of neuroblastoma.

The frequency and copy numbers of gene amplification of ALK were significantly lower in neuroblastic tumors compared with neuroblastic cell lines. Remarkable amplification of the *ALK* gene was detected in 1 tumor tissue of 85 tumor samples examined. Three neuroblastoma cell lines with *ALK* amplification had more than 30 copies of *ALK*, whereas primary neuroblastoma containing *ALK* gene amplification had within a range of 2 to 10 copies. This may be due to underestimation of the copy number in the tumor cells because of contamination of stromal cells and lymphocytes into the tumor tissues.^{34,35} There may also be a mechanism in which cells with a higher copy number of *ALK* become the major population during the establishment of cell lines because of their growth advantage. Immunohistochemical analysis demonstrated, however, universal cytoplasmic expression of ALK in a wide range of neuroblastoma tumor samples, suggesting some transcriptional or posttranslational regulation of the ALK amount might exist in neuroblastoma cells. Although, due to the condition of the samples, we were unable to obtain information on the copy numbers of the *ALK* gene as for the samples used in the immunohistochemical analysis, further immunohistochemical screening may reveal neuroblastoma tissues with an outstanding amount of ALK protein because of gene amplification.

The *N-myc* gene was also amplified in this tumor and in all three cell lines with *ALK* amplification (NB-39-nu, Nagai, and NB-1). *N-myc* is located on 2p24.3 and *ALK* is on 2p23.2, suggesting that there is a tendency to synchronic amplification between *N-myc* and *ALK*. We were unable to conclude that there was an association between *ALK* amplification and prognosis mainly due to the limited number of positive samples and the short-term follow-up. Moreover, the *ALK* gene locus appears too far from the *N-myc* gene locus to be within a single amplicon. Further analysis in a greater number of samples with longer follow-up is necessary.

The activation of ALK results in hyperphosphorylation of ShcC in neuroblastoma cells, and NB-39-nu cells treated with *ALK*-siRNAs show suppressed tyrosine phosphorylation of ShcC, followed by apoptotic changes

to these cells, suggesting that ShcC is a physiological substrate of the activated ALK kinase and that the ALK-ShcC pathway dominantly controls the survival of NB-39-nu cells even with the existence of other RTKs, such as EGFR, TrkA, and Ret. In neuronal cells, both ShcB (Sli/SCK) and ShcC (Rai/N-Shc) can bind activated RTKs, including the EGFR and Trk receptor.³⁶⁻³⁹ Mice lacking both ShcB and ShcC exhibit a significant loss of sympathetic neurons, suggesting that ShcB and ShcC act in supporting sympathetic development and survival.²⁸ A recent study also showed that ShcC is a physiological substrate of Ret kinase and that it exerts a prosurvival function in neuronal cells.⁴⁰ Although high levels of TrkA expression correlate with a favorable outcome of neuroblastoma patients,²⁰ TrkA expression was significantly high in NB-39-nu and Nagai, which derive from tumors with a poor prognosis. This discrepancy may also be explained by the overwhelming control of cell survival by ALK kinase in these cell lines. Neuronal apoptosis is regulated through the action of critical protein kinase cascades, such as the phosphatidylinositol 3-kinase/Akt pathway and the Ras-MAPK pathway.^{41,42} Apparently, neither pathway is properly controlled by EGF or nerve growth factor in NB-39-nu cells or Nagai cells.²⁷ Here, we also demonstrated that the suppression of activated ALK blocks MAPKs and Akt in these cells, resulting in apoptosis. On the other hand, the activity of MAPKs and Akt was not reduced by the suppression of a single copy of *ALK* in SK-N-MC cells. These results suggest that activation of ALK kinase completely remodeled the cellular signaling transduction pathways through ShcC so that cell survival entirely depended on signals originating from ALK kinase.

In conclusion, phosphorylation of several signaling molecules and cancer survival might be under the control of activated ALK kinase when gene amplification of ALK is as significant as in NB-39-nu cells, although the frequency of gene amplification in neuroblastoma tissues is not high. Cytoplasmic expression of ALK in neuroblastoma cells may suggest distinct function of this kinase in cell proliferation and survival. These findings further suggest that activated ALK kinase will be indispensable information for prognosis and treatment of neuroblastoma although the frequency is low.

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Functional implication of p73 protein stability in neuronal cell survival and death

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Abstract

p73, a newly identified member of p53 family, locates at human chromosome 1p36.2-3, a region which is frequently deleted in a wide variety of human tumors including neuroblastoma. p73 is induced to be accumulated in response to a subset of DNA damaging agents such as cisplatin, and thereby promoting G1/S cell cycle arrest and/or apoptosis. Since the expression levels of p73 are kept extremely low under normal conditions, stabilization of p73 is critical for its effects on cell growth inhibition and apoptosis. Indeed, p73 is induced at protein level in SH-SY5Y neuroblastoma cells exposed to cisplatin. Several lines of evidence indicate that stress-induced post-translational modifications of p73 such as phosphorylation and acetylation lead to a marked extension of its half-life. p73 stability is regulated at least in part by proteasome-dependent degradation pathway, however, MDM2 which mediates ubiquitination and subsequent degradation of p53 by the 26S proteasome, does not promote the proteolytic degradation of p73, implying that the protein stability of p73 is regulated through a pathway distinct from that of p53. Although little is known about the regulation of p73 turnover, we are now beginning to understand the regulatory mechanisms by which p73 is induced to be stabilized in response to apoptotic stimuli, and exerts its pro-apoptotic activity. In this review, we discuss about the cellular proteins implicated in the stability control of p73.

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Keywords: E3 ubiquitin ligase; MDM2; Neuroblastoma; p53; p73; p63; Proteasome; Ubiquitination; UFD2a

1. Introduction

p73 belongs to the tumor suppressor p53 family including p53, p73 and p63 [1,2]. As expected from their structural similarity, particularly in the central

sequence-specific DNA-binding domain (over 60% amino acid sequence identity), p73 displays several p53-like properties. Analogous to p53, p73 can transactivate a large number of p53-responsive genes such as *p21^{WAF1}* and *Bax*, and thereby inducing G1/S cell cycle arrest and/or apoptosis in a variety of cancerous cells [3]. p73 gene has been mapped to human chromosome 1p36.2-3, a region which is frequently lost in neuroblastoma and other types of

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tumors [1]. In a sharp contrast to p53, however, p73 is rarely mutated in human tumors including neuroblastoma despite an extensive search [4], and the loss of p73 does not predispose mice to cancer [5], suggesting that p73 does not function as a classic Knudson-type tumor suppressor. Recently, it has been demonstrated that the p53-dependent apoptosis requires the indirect contribution of at least one of the other p53 family members, p73 or p63 [6], whereas p73 is sufficient to induce apoptosis in the absence of p53 [7,8]. Thus, it is likely that p73 cooperates with p53 to induce apoptosis and/or exerts its pro-apoptotic activity in a p53-independent manner. These findings have emphasized the functional importance of p73 in the regulation of apoptotic response, and attracted considerable attention. In particular, p73 is regarded as an important cell fate determinant of neuroblastoma in response to apoptotic stimuli, because wild-type p53 lacks its intact function due to its abnormal cytoplasmic localization in neuroblastoma [9].

Unlike p53, p73 is expressed as multiple variants with varying COOH-terminal extensions (TA isoforms) and lacking NH₂-terminal transactivation domain (Δ N isoforms), arising from alternative splicing and promoter usage, respectively (Fig. 1) [3]. Among them, Δ Np73 has an oncogenic potential [10], and exhibits a dominant-negative behavior toward TAp73 as well as p53 [5,11]. Consistent

with this notion, the expression levels of Δ Np73 are closely associated with poor prognosis in human tumors including neuroblastoma [12,13]. Intriguingly, we and others found that p73 directly transactivates the expression of Δ Np73, suggesting that there exists a negative feedback regulation of p73 by Δ Np73 to modulate cell survival and death [14,15].

Steady-state expression levels of endogenous p73 are maintained at extremely low level under normal conditions, keeping this dangerous protein in an inactive state. In response to a subset of genotoxic stresses including oncotoxic drug cisplatin and ionizing radiation, however, p73 is induced to be accumulated at protein level, and the stabilization of p73 results in either G1/S cell cycle arrest or commitment to death through apoptosis [16]. Thus, stabilization of p73 is directly linked with its function. Several pieces of evidence suggest that the protein stability of p73 is regulated through a pathway distinct from that of p53 [8,17]. Although p73 protein stability is regulated at least in part by proteasomal degradation [17,18], it remains still unknown whether the proteasome-mediated degradation system is the main degradation route of p73. In this review, we will discuss about the cellular proteins regulating the p73 stability and how they affect its stability and activity.

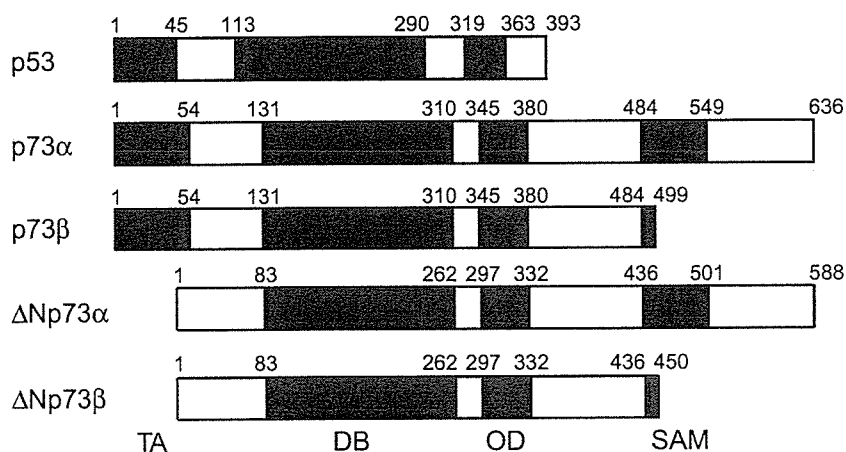


Fig. 1. Structural comparison between p53 and p73. p73 α and p73 β are generated by alternative splicing. Alternative promoter usage gives rise to Δ Np73 α and Δ Np73 β . The domains indicated are: a transactivation domain (TA), a DNA-binding domain (DB), an oligomerization domain (OD) and a sterile alpha motif domain (SAM).

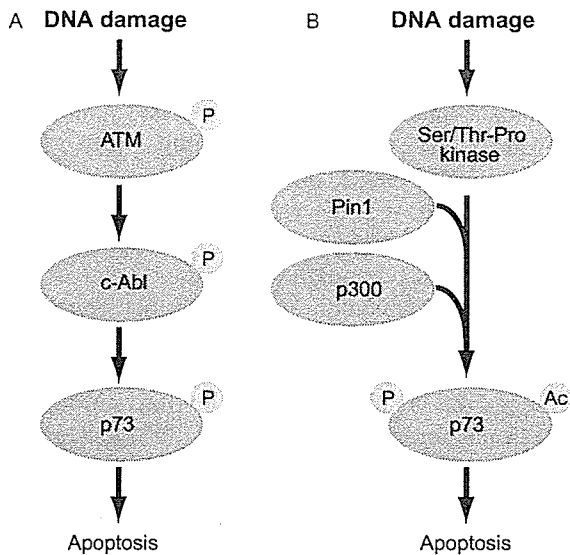


Fig. 2. Chemical modification enhances the stability as well as pro-apoptotic activity of p73 in response to DNA damage. (A) In response to DNA damage, nuclear nonreceptor tyrosine kinase c-Abl is activated by ATM, and thereby phosphorylates p73 at Tyr-99. (B) Pin1 recognizes phosphorylated serine or threonine residues of p73 which can be catalyzed by as yet unidentified Ser/Thr-Pro-directed kinase(s), and enhances its acetylation by p300.

2. Chemical modifications which make p73 stable

Genotoxic stresses including cisplatin treatment and ionizing radiation prolong the half-life of p73 and enhance its pro-apoptotic activity in a pathway dependent on nuclear nonreceptor tyrosine kinase c-Abl [19–21]. According to those results, c-Abl interacts with p73 through the SH3 domain of c-Abl and the p73 PXXP motif, and directly phosphorylates p73 at Tyr-99. Consistent with this notion, p73 is not induced to be accumulated in c-Abl-deficient cells in response to cisplatin (Fig. 2A) [19]. Intriguingly, Ben-Yehoyada et al. found that ionizing radiation induces the tyrosine phosphorylation as well as stabilization of p73 in a c-Abl-dependent manner, and the phosphorylated forms of p73 become associated with the nuclear matrix, suggesting that c-Abl-mediated nuclear redistribution of p73 might play a critical role in the regulation of p73 function [22]. They also described that the amounts of p73 associated with nuclear matrix are increased in the presence of proteasome inhibitor such as MG-132. Alternatively, cisplatin treatment promotes the interaction between

p73 and protein kinase C δ catalytic fragment (PKC δ CF) [23]. PKC δ CF phosphorylates p73 at Ser-289, and increases its stability. Since PKC δ CF enhances the catalytic activity of c-Abl, it is likely that c-Abl could act as a second signal in the functional interaction with p73. Recently, Gonzalez et al. reported that cisplatin-induced apoptosis is associated with the phosphorylation of p73 at Ser-47 catalyzed by Chk1 [24]. Chk1-mediated phosphorylation might convert a latent form of p73 to an active one. Additionally, p73 is phosphorylated at Thr-86 by CDK complexes in a cell cycle-dependent manner [25]. In a sharp contrast to Chk1, the CDK-mediated phosphorylation causes a significant inhibition of the transcriptional activity of p73. Under their experimental conditions, CDK complexes have negligible effect on the amounts of p73.

In addition to the DNA-damage-induced phosphorylation of p73, p73 is regulated by acetylation. Previously, it has been demonstrated that p300 interacts with the NH₂-terminal region of p73, and enhances its pro-apoptotic activity [26]. It is worth noting that doxorubicin treatment induces the acetylation of p73 at Lys-321, Lys-327 and Lys-331 and this acetylation is mediated by p300 in a c-Abl-dependent manner [27]. Based on these results, acetylated form of p73 has a pro-apoptotic activity, whereas nonacetylatable p73 fails to promote apoptosis. Moreover, Mantovani et al. found that p300-mediated acetylation of p73 is enhanced by prolyl isomerase Pin1, and promotes the stabilization of p73 [28]. Pin1 binds to the phosphorylated serine or threonine residue followed by proline of p73, and induces its conformational shift to stimulate the interaction between p73 and p300 (Fig. 2B). Thus, the identification of Ser/Thr kinase(s) required for the phosphorylation of Pin1-binding site on p73 is important to clarify a molecular mechanism underlying the DNA damage-induced stabilization of p73.

3. Ubiquitin-mediated proteolysis of p73

p73 level is dependent on a balance between protein synthesis and degradation. As described by Lee and La Thangue [18], the steady-state expression levels of p73 are significantly increased in the presence of proteasome inhibitor such as LLnL,

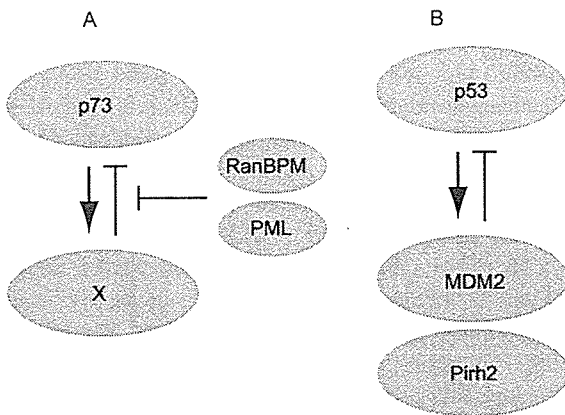


Fig. 3. Ubiquitination-dependent degradation pathway of p73 is distinct from that used for p53. (A) Ubiquitination of p73 could be mediated by p73-induced gene product(s), and significantly inhibited by RanBPM and/or PML. (B) p53-induced E3 ubiquitin ligases MDM2 and Pirh2 promote the ubiquitination and subsequent degradation of p53 by 26S proteasome, however, they have negligible effect on the ubiquitination level of p73.

suggesting that p73 turnover is regulated at least in part by a ubiquitin-dependent proteasome pathway. They also showed that the COOH-terminal extension of p73 α is involved in the regulation of protein stability through a pathway that is sensitive to proteasome inhibitor. Recently, we found that RanBPM binds to the extreme COOH-terminal region of p73 α , and inhibits its ubiquitination [29]. Of note, Bernassola et al. reported that PML promotes the p300-mediated acetylation of p73, and reduces its ubiquitination levels by competition between acetylation and ubiquitination [30]. In addition, Wu et al. described that the transcriptional activity of p73 is required for its rapid degradation, indicating that the direct transcriptional target(s) of p73 could have an ability to induce its proteolytic degradation (Fig. 3A) [31]. In accordance with this notion, Δ Np73 which lacks the NH₂-terminal transactivation domain, is much more stable than wild-type p73. MDM2 which acts as an E3 ubiquitin ligase for p53, promotes the ubiquitination and subsequent degradation of p53 [32–34]. MDM2 is transcriptionally activated by p53, forming an autoregulatory feedback loop with p53 to tightly regulate its expression levels (Fig. 3B). Similar to p53, p73 stimulates the transcription of MDM2. MDM2 interacts with the NH₂-terminal transactivation domain of p73 and abrogates its transcriptional

activity as well as pro-apoptotic function, whereas it fails to mediate the proteolytic degradation of p73 [35,36]. Like p73, p63 is degraded by a proteasome-dependent pathway, however, MDM2 increases the steady-state level of intracellular p63 [37]. Another p53-induced E3 ubiquitin ligase Pirh2 which promotes p53 degradation (Fig. 3B), has no detectable effect on p73 stability [31,38], suggesting that p73 degradation might be mediated by an as yet unidentified E3 ubiquitin ligase(s) distinct from that used for p53 degradation. Intriguingly, Toh et al. described that c-Jun fails to interact with p73 but prolongs the half-life of p73 by preventing its proteasome-dependent degradation [39]. According to those results, the transactivation function of c-Jun is required for the c-Jun-mediated stabilization of p73. Thus, it is likely that c-Jun activates its transcriptional target(s) implicated in the regulation of p73 stability.

4. Ubiquitination-independent degradation of p73

In addition to the ubiquitin-mediated proteasome pathway, p73 degradation is regulated in a ubiquitination-independent manner. Cyclin G which belongs to the cyclin family, has been shown to be one of the transcriptional target genes of p53 [40]. Recently, Ohtsuka et al. demonstrated that cyclin G interacts with p73 and promotes its down-regulation at protein level [41]. siRNA-mediated knock-down of the endogenous cyclin G results in a significant accumulation of p73 in response to DNA damage. Unexpectedly, cyclin G does not increase the amounts of the ubiquitinated forms of p73, suggesting that cyclin G-mediated destabilization of p73 might be regulated through a pathway distinct from the ubiquitination-dependent degradation system. However, the precise regulatory mechanism of p73 stability by cyclin G remains to be elusive.

5. Viral oncoproteins

As described by Marin et al. [42], HEK293 human embryonic kidney and COS monkey kidney cells express the higher levels of p73 as compared with the other cell lines. HEK293 and COS cells are transformed with viral oncoproteins such as adenovirus E1B and

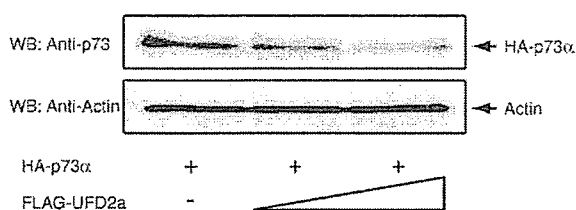


Fig. 4. Down-regulation of p73 by UFD2a. U2OS cells were transiently co-transfected with the indicated combinations of the expression plasmids. Forty-eight hours after transfection, whole cell lysates were prepared and subjected to Western blot analysis (WB) with the indicated antibodies.

simian virus 40 (SV40) T antigen, respectively. In addition, Lemasson and Nyborg reported that HTLV-1-transformed cells express a high level of p73, whereas p73 is undetectable in uninfected T-cell lines [43]. Viral oncoprotein Tax which is encoded by HTLV-I, has an ability to enhance the stability of p73. Since these viral oncoproteins do not interact directly with p73, as yet unidentified indirect mechanism(s) appears to contribute to maintain the higher levels of p73 in those transformed cells. Considering that adenovirus E1A binds to p300 and inhibits its E3/E4 ubiquitin ligase activity to stabilize p53 [44], it is plausible that viral oncoproteins such as E1B, T antigen and Tax could inhibit the enzymatic activity of unidentified E3/E4 ubiquitin ligase for p73 through the physical interaction, and thereby protecting p73 from ubiquitination-mediated proteolytic degradation.

6. Interaction between p73 and UFD2a in human neuroblastoma cell lines

We have previously identified a homozygously deleted region at 1p36.2-p36.3 in neuroblastoma cell line NB-C201 [45]. Within this region, there exist six genes including *DFF45*, *PGD*, *CORT*, *UFD2a*, *KIF1B-β* and *PEX14*. Among them, UFD2a which belongs to the U-box-type ubiquitin ligase family, has an E3 as well as E4 ubiquitin ligase activity [46,47]. It has been shown that UFD2a is implicated in cell survival under a certain stress condition [46]. In response to apoptotic stimuli, UFD2a is cleaved by caspase 6 or granzyme B, and its enzymatic activity is markedly impaired [48]. It is, thus, tempting to speculate that UFD2a might participate in the regulation of cell survival and death.

Recently, we have found that, during the cisplatin-mediated apoptosis in SH-SY5Y neuroblastoma cells, UFD2a is down-regulated at protein level in association with a significant accumulation of p73. Ectopic expression of UFD2a results in a reduction of p73 (Fig. 4), and this observation is supported by a decrease in a half-life of p73 in UFD2a-expressing cells. This is consistent with our additional findings showing that UFD2a inhibits the p73-mediated transcriptional activation and apoptosis [Hosoda et al., manuscript in preparation]. Although it is not known whether UFD2a-mediated degradation of p73 could be regulated in a ubiquitination-dependent manner, it is conceivable that down-regulation of UFD2a in response to cisplatin might augment the p73-dependent apoptosis, and thereby providing a novel strategy for anticancer treatment.

7. Conclusion

In contrast to other types of human tumors, p53 is rarely mutated in neuroblastoma, however, the aberrant cytoplasmic retention of wild-type p53 renders it nonfunctional. Since p73 has an ability to induce tumor cell apoptosis irrespective of p53 status, it is likely that p73 is a pivotal player in apoptotic response, particularly in tumors such as neuroblastoma lacking functional p53. Accumulating evidence strongly suggests that p73 is regulated through a pathway distinct from that of p53. In this respect, it is necessary to clarify signaling pathways which specifically regulate the activity as well as stability of p73 in response to apoptotic stimuli.

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p73, a sophisticated p53 family member in the cancer world

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p73 belongs to a family of p53-related nuclear transcription factors that includes p53, p73 and p63. The overall structure and sequence homology indicates that a p63/p73-like protogene is the ancestral gene, whereas p53 evolved later in higher organisms. In accordance with their structural similarity, p73 functions in a manner analogous to p53 by inducing tumor cell apoptosis and participating in the cell cycle checkpoint control through transactivating an overlapping set of p53/p73-target genes. In sharp contrast to p53, however, p73 is expressed as two NH₂-terminally distinct isoforms including transcriptionally active (TA) and transcriptionally inactive (Δ N) forms. Δ Np73, which has oncogenic potential, acts in a dominant negative manner against TAp73 as well as p53. p73 is induced to be stabilized in response to a subset of DNA-damaging agents in a way that is distinct from that of p53, and exerts its pro-apoptotic activity. Several lines of evidence suggest that p73 can induce tumor cell apoptosis in a p53-dependent and p53-independent manner. Some tumors exhibit resistance to the p53-dependent apoptotic program, therefore p73, which can induce apoptotic cell death by p53-independent mechanisms, is particularly useful. In this review, we discuss the regulatory mechanisms of p73 activity, and also the functional significance of p73 in the regulation of cellular processes including tumorigenesis, apoptosis and neurogenesis. (*Cancer Sci* 2005; 96: 729–737)

Until recently, the tumor suppressor p53 has been believed to be encoded by a single gene which lacks any structural or functional homologs. The identification of two p53-related proteins, termed p73 and p63, revealed that p53 belongs to a small family of sequence-specific nuclear transcription factors.^(1–3) p53 family members share three major functional domains: the NH₂-terminal transactivation domain; the central core sequence-specific DNA-binding domain; and the COOH-terminal oligomerization domain. Of these, the central DNA-binding domain is highly conserved across the family. As expected from their structural similarities, p73 can bind to the p53-responsive elements, and transactivate an overlapping set of p53-target genes implicated in G1/S cell cycle arrest and apoptotic cell death.^(1,4) Recent studies demonstrated that p73 is required for p53-dependent apoptosis.⁽⁵⁾ Unlike p53, p73 is expressed as at least six variants with different COOH-terminal ends, arising from the alternative splicing at the 3' portion of the primary transcript.^(1,6,7) Each of these splicing variants (TAp73) contains an intact NH₂-terminal transactivation domain, and exerts its transcriptional activity to various degrees. Additionally, p73 contains a second

transcriptional start site within intron 3, giving rise to the NH₂-terminally truncated form of p73 (Δ Np73) which has little transcriptional activity.⁽⁸⁾ Similar to p73, Δ Np63 is also generated by an alternative promoter (Fig. 1a,b).⁽²⁾ Δ Np73 displays dominant negative behavior toward p73 as well as wild-type p53, and has oncogenic potential.^(9,10) Of note, we and others found that Δ Np73 is a direct transcriptional target of p73, suggesting that there exists a negative feedback regulation of p73 by Δ Np73, to modulate cell survival and death.^(11–13)

Steady-state expression levels of endogenous p73 are kept extremely low under physiological conditions. Similar to p53, p73 is induced to be stabilized at the protein level in response to a subset of DNA-damaging agents, and exerts its pro-apoptotic activity.⁽¹⁴⁾ Accumulating evidence suggests that p73 turnover is regulated through a ubiquitination-dependent and ubiquitination-independent degradation pathway. MDM2 acts as an E3 ubiquitin protein ligase for p53, and promotes the proteasome-mediated proteolytic degradation of p53.^(15–17) On the other hand, MDM2 increases the stability of p73,⁽¹⁸⁾ indicating that p73 stability is regulated through a pathway distinct from that of p53. Alternatively, Ohtsuka *et al.* reported that cyclin G binds to p73 and stimulates its proteolytic degradation in a ubiquitination-independent manner, however, the precise molecular mechanism of cyclin G-mediated degradation of p73 remains unknown.⁽¹⁹⁾

Considering that p73 has a p53-like property and is mapped to the human chromosome 1p36.2-3, a region which is frequently lost in a wide variety of human tumors including neuroblastoma, it is likely that p73 could be one of the classic Knudson-type tumor suppressors.⁽¹⁾ In spite of extensive mutation searches, p73 was rarely mutated in primary tumors.⁽²⁰⁾ Additionally, initial genetic studies demonstrated that p73-deficient mice exhibit severe developmental defects, however, they do not develop spontaneous tumors, suggesting that p73 might participate in the regulation of normal development *in vivo*, and that p73 does not link directly to tumor suppression.⁽⁸⁾ Indeed, p73 has the ability to induce neuronal differentiation of undifferentiated neuroblastoma cells.⁽²¹⁾ However, this viewpoint has been challenged by the observation that mice mutant for p73 and p63 develop spontaneous tumors, and their spectrum is quite different from that of p53-deficient mice.⁽²²⁾ Thus, it is likely that p73 and

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Abbreviations: EEC, ectrodactyly, ectodermal dysplasia, and facial clefts (syndrome); OPC, oligodendrocyte precursor cell; SAM, sterile α motif; YAP, Yes-associated protein.

As described above, Δ Np73 is transcriptionally inactive due to a lack of the NH₂-terminal transactivation domain in TAp73. However, this viewpoint has been challenged by the recent finding of Liu *et al.* showing that Δ Np73 β has weak but distinct transcriptional activity, thereby inducing cell cycle arrest and/or apoptosis.⁽²⁹⁾ In contrast to Δ Np73 β , Δ Np73 α failed to induce cell cycle arrest and/or apoptosis under their experimental conditions. According to their results, the NH₂-terminal 13 unique amino acid residues as well as PXXP motifs of Δ Np73 β might be a novel activation domain. Thus, it is possible that Δ Np73 β might exert a distinct function under certain cellular processes.

Transcriptional regulation of the main promoter of p73

E2F1 transcription factor plays an important role in the regulation of cell cycle progression by inducing the transcription of genes whose products are directly or indirectly required for entry into the S phase.⁽³⁰⁾ In addition to the proliferative effect of deregulated E2F1 activity, unscheduled E2F1 activation leads to apoptosis to protect cells from cellular transformation.⁽³¹⁾ Consistent with this notion, E2F1-deficient mice exhibited a high incidence of unusual tumors.^(32,33) E2F1-induced apoptosis is regulated in a p53-dependent or p53-independent manner. It is interesting that the p73 promoter region contains a TATA-like box and at least three E2F1-binding sites, and indeed the enforced expression of E2F1 strongly stimulates the transcription of p73 through the direct binding to the E2F1-responsive elements in the p73 promoter.^(34,35) The E2F1-mediated up-regulation of p73 results in a significant induction of apoptosis. Other studies demonstrated that T cell receptor-mediated apoptosis is dependent on both E2F1 and p73.⁽³⁶⁾ Thus, E2F1-mediated apoptosis requires p73, at least in part. Alternatively, E2F1 might also contribute to the up-regulation of p73 mRNA levels during muscle and neuronal differentiation of murine C2C12 myoblasts and P19 cells, respectively.⁽³⁷⁾ It is worth noting that Chk1 and Chk2 are required for the induction of p73 in response to DNA damage, and E2F1 contributes to the Chk kinase-dependent transcriptional regulation of p73.⁽³⁸⁾ In addition to E2F1, cellular and viral oncogene products such as c-Myc and E1A indirectly activated the transcription of p73.⁽³⁹⁾

Recently, Fontemaggi *et al.* identified a 1 kb negative regulatory fragment within the first intron of p73 gene.⁽³⁷⁾ Under their experimental conditions, this intronic fragment significantly reduced the activity of the p73 promoter upon E2F1 overexpression. Of note, the p73 intronic fragment contained six consensus binding sites for transcriptional repressor ZEB. Ectopic expression of ZEB in C2C12 myoblasts attenuated myotube formation, and repressed the transcription of p73. In accordance with these results, the dominant negative form of ZEB had an ability to restore the expression levels of p73 in proliferating cells.

Because DNA hypermethylation contributes to the alteration of the entry of transcription factors into the regulatory region, the epigenetic modification of the p73 promoter region through aberrant hypermethylation could be an alternative molecular mechanism for silencing the p73 gene. Corn

et al. described the aberrant promoter methylation of p73 as occurring frequently in primary acute lymphoblastic leukemias and Burkitt's lymphomas, whereas the p73 promoter methylation was not detected in normal lymphocytes or bone marrow.⁽⁴⁰⁾ Similar results were also reported by Kawano *et al.*⁽⁴¹⁾ In contrast, hypermethylation of the p73 promoter region was not observed in solid tumors including breast, renal, colon cancers or neuroblastomas,⁽⁴²⁾ suggesting that the methylation-dependent silencing of p73 transcription might be specific to hematological malignancies.

p73 is rarely mutated in human cancers

The p73 gene has been mapped to human chromosome 1p36.2-3, a region which exhibits frequent loss of heterozygosity in a wide variety of human cancers including neuroblastoma, and its gene product has an ability to promote G1/S cell cycle arrest and/or cell death through apoptosis in certain cancerous cells. Therefore, p73 could act as a tumor suppressor.⁽¹⁾ In spite of an extensive search of the p73 status in human primary tumors, p73 was infrequently mutated in many human tumors.^(20,43-48) p73 mutations were detected in fewer than 0.5% of human cancers, whereas over 50% of cancers carry p53 mutations. For example, only two types of p73 mutations with amino acid substitution (P405R and P425L) were found in primary neuroblastoma and lung cancer.⁽²⁰⁾ In addition to the NH₂-terminal transactivation domain, Takada *et al.* found a potential second transactivation domain within the COOH-terminal portion of p73 α (amino acid residues 380-513), albeit to a lesser extent than the NH₂-terminal transactivation domain.⁽⁴⁹⁾ This region is rich in glutamine and proline residues. Among the two types of p73 mutations, the P425L substitution significantly reduced the transcriptional and growth-suppressive activity of p73 α , whereas the P405R substitution had a negligible effect on p73 α .⁽⁵⁰⁾

In sharp contrast to p53-deficient mice, which develop tumors with high frequency,⁽⁵¹⁾ p73-deficient mice were viable, but the loss of p73 did not predispose mice to cancer, suggesting that p73 does not function as a classic Knudson-type tumor suppressor,⁽⁸⁾ and its possible contribution to tumor suppression is still unclear. Instead, mice lacking p73 displayed severe developmental defects, including hydrocephalus, hippocampal dysgenesis, and abnormalities in the pheromone sensory pathways. These observations strongly suggest that p73 and p53 have distinct biological functions, and p73 plays an important role in normal development, especially in neural development and apoptosis. Because those p73-deficient mice lacked both TAp73 and Δ Np73 variants, further studies of variant-specific knockout mice might provide an insight into the unique role of each variant in tumorigenesis.

As mentioned above, initial genetic studies revealed that p73-deficient mice do not display an increased susceptibility to spontaneous tumorigenesis. More recently, Flores *et al.* examined whether synergistic effects of p73 and p63 could exist, alone or in combination with p53, in tumor suppression.⁽²²⁾ Strikingly, they found that p73 and p63 heterozygous mice (p73^{+/-} and p63^{+/-}) developed malignant tumors at high frequency including various tumor types not observed in p53^{+/-} mice, and p53^{+/-}; p73^{+/-} and p53^{+/-}; p63^{+/-} mice

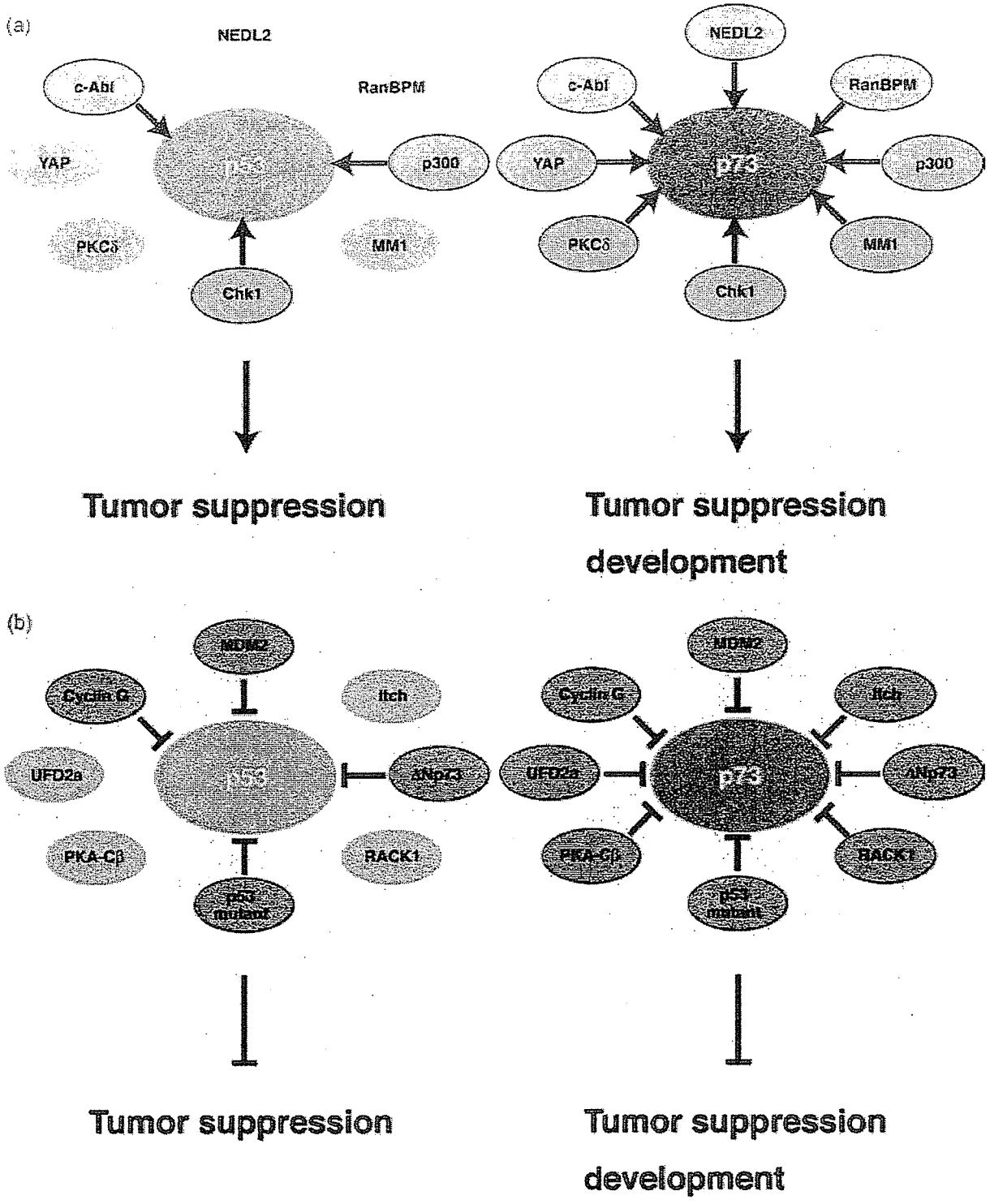


Fig. 4. Positive (a) and negative (b) regulation of p73 or p53 activity through the physical and functional interaction with various cellular proteins. Uncircled proteins have an undetectable effect on p53.

NH₂-terminal transactivation domain, and subsequent proteolytic degradation of p53.⁽¹⁵⁻¹⁷⁾ Similar to p53, MDM2 was also a direct transcriptional target of p73, bound to its NH₂-terminal transactivation domain and thereby inhibiting

p73-mediated transcriptional activation and apoptosis. However, MDM2 failed to ubiquitinate p73, and this interaction resulted in an increase in p73 protein stability.⁽¹⁸⁾ Additionally, a newly identified p53-induced E3 ubiquitin