Tumorigenesis and Neoplastic Progression

Biological Role of Anaplastic Lymphoma Kinase in Neuroblastoma

Yuko Osajima-Hakomori,^{*¶||} Izumi Miyake,^{*†}
Miki Ohira,[‡] Akira Nakagawara,[‡]
Atsuko Nakagawa,[§] and Ryuichi Sakai^{*}

From the Growth Factor Division,^{*} National Cancer Center Research Institute, Chuo-ku, Tokyo; St. Marianna University School of Medicine,[¶] Kawasaki-shi, Kanagawa; Tokyo Metropolitan Geriatric Hospital,^{||} Itabashi-ku, Tokyo; the Department of Pediatrics,[†] Kitasato University School of Medicine, Sagami-ku, Kanagawa; the Division of Biochemistry,[‡] Chiba Cancer Center Research Institute, Cyuo-ku, Chiba; and the Department of Pathology,[§] Aichi Medical University, Aichi-gun, Aichi, Japan

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-

Supported by the Program for the Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research of Japan. Y.O.-H. is the recipient of a Research Resident Fellowship from the Foundation for Promotion of Cancer Research, Japan.

Accepted for publication March 23, 2005.

Address reprint requests to Ryuichi Sakai, M.D., Growth Factor Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan. E-mail: rsakai@gan2.res.ncc.go.jp.

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 50l 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.

Immunocytochemical Staining

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 *EcoRI* 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'-GAGU-CUGGCAGUUGACUUCdTdT-3' for *ALK*-siRNA1 and 5'-GCUCCGCGUGCCAAGCAGdTdT-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 *Eco*RI. 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.

References

1. Ullrich A, Schlessinger J: Signal transduction by receptors with tyrosine kinase activity. *Cell* 1990, 61:203–212
2. Heldin CH: Dimerization of cell surface receptors in signal transduction. *Cell* 1995, 80:213–223
3. Blume-Jensen P, Hunter T: Oncogenic kinase signalling. *Nature* 2001, 411:355–365
4. Pawson T: Protein modules and signalling networks. *Nature* 1995, 373:573–580
5. Kozlowski M, Larose L, Lee F, Le DM, Rottapel R, Siminovich KA: SHP-1 binds and negatively modulates the c-Kit receptor by interaction with tyrosine 569 in the c-Kit juxtamembrane domain. *Mol Cell Biol* 1998, 18:2089–2099
6. Morris SW, Kirstein MN, Valentine MB, Dittmer KG, Shapiro DN, Saltman DL, Look AT: Fusion of a kinase gene *ALK*, to a nucleolar protein gene *NPM*, in non-Hodgkin's lymphoma. *Science* 1994, 263:1281–1284

7. Shiota M, Fujimoto J, Semba T, Satoh H, Yamamoto T, Mori S: Hyperphosphorylation of a novel 80 kDa protein-tyrosine kinase similar to Ltk in a human Ki-1 lymphoma cell line, AMS3. *Oncogene* 1994, 9:1567-1574
8. Bridge JA, Kanamori M, Ma Z, Pickering D, Hill DA, Lydiatt W, Lui MY, Colleoni GW, Antonescu CR, Ladanyi M, Morris SW: Fusion of the ALK gene to the clathrin heavy chain gene, CLTC, in inflammatory myofibroblastic tumor. *Am J Pathol* 2001, 159:411-415
9. Iwahara T, Fujimoto J, Wen D, Cupples R, Bucay N, Arakawa T, Mori S, Ratzkin B, Yamamoto T: Molecular characterization of ALK, a receptor tyrosine kinase expressed specifically in the nervous system. *Oncogene* 1997, 14:439-449
10. Morris SW, Naeve C, Mathew P, James PL, Kirstein MN, Cui X, Witte DP: 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, 14:2175-2188
11. Ben-Neriah Y, Bauskin AR: Leukocytes express a novel gene encoding a putative transmembrane protein-kinase devoid of an extracellular domain. *Nature* 1988, 333:672-676
12. Maru Y, Hirai H, Takaku F: Human ltk: gene structure and preferential expression in human leukemic cells. *Oncogene Res* 1990, 5:199-204
13. Bernards A, de la Monte SM: The ltk receptor tyrosine kinase is expressed in pre-B lymphocytes and cerebral neurons and uses a non-AUG translational initiator. *EMBO J* 1990, 9:2279-2287
14. Pulford K, Lamant L, Morris SW, Butler LH, Wood KM, Stroud D, Delsol G, Mason DY: Detection of anaplastic lymphoma kinase (ALK) and nucleolar protein nucleophosmin (NPM)-ALK proteins in normal and neoplastic cells with the monoclonal antibody ALK1. *Blood* 1997, 89:1394-1404
15. Shiota M, Fujimoto J, Takenaga M, Satoh H, Ichinohasama R, Abe M, Nakano M, Yamamoto T, Mori S: Diagnosis of t(2;5)(p23;q35)-associated Ki-1 lymphoma with immunohistochemistry. *Blood* 1994, 84:3648-3652
16. Duyster J, Bai RY, Morris SW: Translocations involving anaplastic lymphoma kinase (ALK). *Oncogene* 2001, 20:5623-5637
17. Stoica GE, Kuo A, Aigner A, Sunitha I, Souttou B, Malerczyk C, Caughey DJ, Wen D, Karavanov A, Riegel AT, Wellstein A: Identification of anaplastic lymphoma kinase as a receptor for the growth factor pleiotrophin. *J Biol Chem* 2001, 276:16772-16779
18. Stoica GE, Kuo A, Powers C, Bowden ET, Sale EB, Riegel AT, Wellstein A: Midkine binds to anaplastic lymphoma kinase (ALK) and acts as a growth factor for different cell types. *J Biol Chem* 2002, 277:35990-35998
19. Evans AE, D'Angio GJ, Randolph J: A proposed staging for children with neuroblastoma: children's cancer study group A. *Cancer* 1971, 27:374-378
20. 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, 328:847-854
21. Nakagawara A, Azar CG, Scavarda NJ, Brodeur GM: Expression and function of TRK-B and BDNF in human neuroblastomas. *Mol Cell Biol* 1994, 14:759-767
22. Nakagawara A, Nakamura Y, Ikeda H, Hiwasa T, Kuida K, Su MS, Zhao H, Nnaan A, Sakiyama S: High levels of expression and nuclear localization of interleukin-1 beta converting enzyme (ICE) and CPP32 in favorable human neuroblastomas. *Cancer Res* 1997, 57:4578-4584
23. Posmantur R, McGinnis K, Nadimpalli R, Gilbertsen RB, Wang K: Characterization of CPP32-like protease activity following apoptotic challenge in SH-SY5Y neuroblastoma cells. *J Neurochem* 1997, 68:2328-2337
24. Adida C, Berrebi D, Peuchmaur M, Reyes-Mugica M, Altieri DC: Anti-apoptosis gene, survivin, and prognosis of neuroblastoma. *Lancet* 1998, 351:882-883
25. Hiyama E, Hiyama K, Yokoyama T, Matsuura Y, Piatyszek MA, Shay JW: Correlating telomerase activity levels with human neuroblastoma outcomes. *Nat Med* 1995, 1:249-255
26. Lamant L, Pulford K, Bischof D, Morris SW, Mason DY, Delsol G, Mariame B: Expression of the ALK tyrosine kinase gene in neuroblastoma. *Am J Pathol* 2000, 156:1711-1721
27. Miyake I, Hakomori Y, Shinohara A, Gamou T, Saito M, Iwamatsu A, Sakai R: Activation of anaplastic lymphoma kinase is responsible for hyperphosphorylation of ShcC in neuroblastoma cell lines. *Oncogene* 2002, 21:5823-5834
28. Sakai R, Henderson JT, O'Bryan JP, Elia AJ, Saxton TM, Pawson T: The mammalian ShcB and ShcC phosphotyrosine docking proteins function in the maturation of sensory and sympathetic neurons. *Neuron* 2000, 28:819-833
29. Perucho M, Goldfarb M, Shimizu K, Lama C, Fogh J, Wigler M: Human-tumor-derived cell lines contain common and different transforming genes. *Cell* 1981, 27:467-476
30. Brodeur GM, Pritchard J, Berthold J, Carlsen NL, Castel V, Castellberry RP, De Bernardi B, Evans AE, Favrot M, Hedborg F, Kaneko M, Kemshead J, Lampert F, Lee RE, Look AT, Pearson AD, Philip T, Roald B, Sawada T, Seeger RC, Tsuchida Y, Voute PA: Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment. *J Clin Oncol* 1993, 11:1466-1477
31. Shimada H, Ambros IM, Dehner LP, Hata J, Joshi VV, Roald B, Stram DO, Gerbing RB, Lukens JN, Matthay KK, Castleberry RP: The International Neuroblastoma Pathology Classification (the Shimada system). *Cancer* 1999, 86:364-372
32. Ikeda I, Ishizaka Y, Tahira T, Suzuki T, Onda M, Sugimura T, Nagao M: Specific expression of the ret proto-oncogene in human neuroblastoma cell lines. *Oncogene* 1990, 5:1291-1296
33. Motegi A, Fujimoto J, Kotani M, Sakuraba H, Yamamoto T: ALK receptor tyrosine kinase promotes cell growth and neurite outgrowth. *J Cell Sci* 2004, 117:3319-3329
34. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL: Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 1987, 235:177-182
35. Tsuda H, Hirohashi S, Shimosato Y, Hirota T, Tsugane S, Yamamoto H, Miyajima N, Toyoshima K, Yamamoto T, Yokota J, Yoshida T, Sakamoto H, Terada M, Sugimura T: Correlation between long-term survival in breast cancer patients and amplification of two putative oncogene-coamplification units: hst-1/int-2 and c-erbB-2/ear-1. *Cancer Res* 1989, 49:3104-3108
36. Ganju P, O'Bryan JP, Der C, Winter J, James IF: Differential regulation of SHC proteins by nerve growth factor in sensory neurons and PC12 cells. *Eur J Neurosci* 1998, 10:1995-2008
37. Nakamura T, Komiya M, Gotoh N, Koizumi S, Shibuya M, Mori N: Discrimination between phosphotyrosine-mediated signaling properties of conventional and neuronal Shc adapter molecules. *Oncogene* 2002, 21:22-31
38. Nakamura T, Muraoka S, Sanokawa R, Mori N: N-Shc and Sck, two neuronally expressed Shc adapter homologs: their differential regional expression in the brain and roles in neurotrophin and Src signaling. *J Biol Chem* 1998, 273:6960-6967
39. O'Bryan JP, Songyang Z, Cantley L, Der CJ, Pawson T: A mammalian adaptor protein with conserved Src homology 2 and phosphotyrosine-binding domains is related to Shc and is specifically expressed in the brain. *Proc Natl Acad Sci USA* 1996, 93:2729-2734
40. Pelicci G, Troglio F, Bodini A, Melillo RM, Pettitrossi V, Coda L, De Giuseppe A, Santoro M, Pelicci PG: The neuron-specific Rai (ShcC) adaptor protein inhibits apoptosis by coupling Ret to the phosphatidylinositol 3-kinase/Akt signaling pathway. *Mol Cell Biol* 2002, 22:7351-7363
41. Yuan J, Yankner BA: Apoptosis in the nervous system. *Nature* 2000, 407:802-809
42. De Vita G, Melillo RM, Carlomagno F, Visconti R, Castellone MD, Bellacosa A, Billaud M, Fusco A, Tschlis PN, Santoro M: Tyrosine 1062 of RET-MEN2A mediates activation of Akt (protein kinase B) and mitogen-activated protein kinase pathways leading to PC12 cell survival. *Cancer Res* 2000, 60:3727-3731