Midkine Promotes Neuroblastoma through Notch2 Signaling

Satoshi Kishida1, Ping Mu1, Shin Miyakawa2, Masatoshi Fujiwara2, Tomoyuki Abe2, Kazuma Sakamoto1, Akira Onishi3, Yoshikazu Nakamura2,3, and Kenji Kadomatsu1

Abstract

Midkine is a heparin-binding growth factor highly expressed in various cancers, including neuroblastoma, the most common extracranial pediatric solid tumor. Prognosis of patients with neuroblastoma in which MYCN is amplified remains particularly poor. In this study, we used a MYCN transgenic model for neuroblastoma in which midkine is highly expressed in precancerous lesions of sympathetic ganglia. Genetic ablation of midkine in this model delayed tumor formation and reduced tumor incidence. Furthermore, an RNA aptamer that specifically bound midkine suppressed the growth of neuroblastoma cells in vitro and in vivo in tumor xenografts. In precancerous lesions, midkine-deficient MYCN transgenic mice exhibited defects in activation of Notch2, a candidate midkine receptor, and expression of the Notch target gene HES1. Similarly, RNA aptamer-treated tumor xenografts also showed attenuation of Notch2-HES1 signaling. Our findings establish a critical role for the midkine-Notch2 signaling axis in neuroblastoma tumorigenesis, which implicates new strategies to treat neuroblastoma. Cancer Res; 73(4); 1318–27. ©2012 AACR.

Introduction

Midkine is a heparin-binding growth factor originally identified in embryonal carcinoma cells and has been implicated in tumor development (1–3). Midkine expression is intensive during the midgestation period and is decreased thereafter (4). Therefore, midkine expression is low in normal adult tissues. However, it is resumed upon the development of various tumors, including Wilms’ tumor (5), gastrointestinal cancer (6), and astrocytoma (7). In human colorectal cancer and prostate cancer, midkine is strongly expressed at the precancerous stages (8, 9). Importantly, knockdown of midkine in a xenograft model with colorectal and prostate cancer cells was associated with significant suppression of tumor growth (10–13). However, it remains elusive whether endogenous midkine is crucial for tumorigenesis. Furthermore, reagents that block the function of secreted midkine are still unavailable for clinical use.

Neuroblastoma is one of the cancers whose pathogenesis seems closely related to midkine expression. Neuroblastoma, which arises from a neural crest-derived sympathetic neuronal lineage, is the most common extracranial pediatric solid tumor (14). It accounts for around 15% of all pediatric cancer-related deaths, and the prognosis still remains poor despite an enormous amount of basic and clinical research. The transcription factor MYCN is one of the most potent predisposition genes for neuroblastoma (14). There are several established prognostic factors for neuroblastoma viz., MYCN amplification, low TRKA expression, diploidy, and age older than 18 months have all been related with poor prognosis (15). In addition, we previously reported that midkine could also be a potent prognostic factor (16, 17). Midkine mRNA is highly expressed in neuroblastoma tissues with poor prognosis, whereas pleiotrophin (PTN), another family member of midkine, is expressed in tumors with good prognosis (18).

As an animal model for neuroblastoma, MYCN transgenic mice, in which the human MYCN gene was integrated under the control of a rat tyrosine hydroxylase promoter, were generated (19). MYCN transgenic mice spontaneously develop tumors mainly from the superior mesenteric ganglion (SMG), one of the sympathetic ganglia. Because those tumors are pathologically equivalent to human neuroblastoma (19), they have been used for the molecular investigation of neuroblastoma tumorigenesis. Hemizygous MYCN transgenic mice show a hyperplasia phenotype in the SMG at the age of 2 weeks, and these hyperplastic cells are thought to be precancerous lesions (20, 21).

RNA aptamers are biochemical or therapeutic agents that directly target a given molecule. An RNA aptamer, which specifically recognizes a particular protein, is selected from a complex library of random RNA sequences typically containing 1014 different molecules through a process known as systematic evolution of ligands by exponential enrichment (SELEX; refs. 22–27). The concept is based on the ability of short (20–80-mer) RNA sequences to fold into unique 3-dimensional structures that recognize a particular target with
high affinity and specificity. Therefore, aptamers can be thought of as nucleic acid analogs to antibodies.

In this study, we investigated the role of endogenous midkine in the tumorigenesis of neuroblastoma. In addition, we examined the therapeutic impact of an RNA aptamer that blocked secreted midkine on neuroblastoma in mice.

Materials and Methods

Cell culture

Human neuroblastoma cell line TNB1, YT-nu, and human prostate adenocarcinoma cell line LNCap were obtained from RIKEN Cell Bank (Tsukuba, Japan), the Carcinogenesis Division, National Cancer Center Research Institute (Tokyo, Japan), and American Type Culture Collection, respectively. They were cultured with RPMI-1640 supplied with 10% heat-inactivated FBS in an incubator with humidified air at 37°C with 5% CO₂. On receiving the cell lines, we prepared the frozen stocks within 1 to 2 passages. In every 1 to 2 months, we thawed the stock to keep original condition. They were routinely authenticated on the basis of viability, growth rate, and morphology by microscopic examination.

Animals

MYCN transgenic mice (19) were maintained in our animal facility under a controlled environment and provided with standard nourishment and water. They were crossed with 129-Ter/SvJcl wild-type mice (CLEA Japan). Their abdomens were inspected for the tumor every week, and mice with the tumor were immediately euthanized with CO₂. Midkine-knockout mice were established as previously described (28). This study was approved by the Animal Care and Use Committee of Nagoya University Graduate School of Medicine (Nagoya, Japan).

Reverse transcription PCR and real-time PCR

RNA extraction from SMG, tumor tissues and cultured cells, and reverse transcription PCR (RT-PCR) were carried out as previously described (29). The annealing temperatures and primer sequences for each gene were shown in Supplementary Table S1. Real-time PCR for midkine was carried out with TaqMan Gene Expression Assays (ABI) and an Mx3005P Real-time QPCR System (Agilent). The assay IDs for midkine and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are Mm00440279_m1 and Mm03302249_g1, respectively. For glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are Mm00440279_m1 and Mm03302249_g1, respectively. For glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are Mm00440279_m1 and Mm03302249_g1, respectively.

RNA aptamers

An RNA aptamer to midkine (77 nucleotides length) was selected by SELEX (23–25). 2′-Fluoro–modified pyrimidines were used for nuclease resistance. This aptamer was truncated to 40 nucleotides and was partially modified with 2′-O-methyl modification (Apt-1). Apt-1 was chemically synthesized with an RNA synthesizer (Gene Design). As a negative control, an RNA, which was composed of scramble sequences of Apt-1 and had much lower affinity to midkine, was also chemically synthesized (Apt-nc).

Immunohistochemistry

Immunohistochemistry was carried out as previously described (29) using following primary antibodies: rabbit anti-midkine (raised against recombinant full-length mouse midkine, 1:1,000), mouse anti-MYCN (OP13, 1:20; Calbiochem), mouse anti-Ki-67 (550609, 1:20; BD Biosciences), rabbit anti-tyrosine hydroxylase (AB152, 1:106; Chemicon), rabbit anti-Notch2 full-length (07-1233, 1:100; Millipore), rabbit anti-Notch2 intracellular domain (ICD; 07-1234, 1:100; Millipore), and rabbit anti-HES1 (AB5702, 1:100; Millipore). Biotin goat anti-rabbit immunoglobulin (Ig; 550338, 1:10; BD Biosciences) and biotin goat anti-mouse Ig (550337, 1:50; BD Biosciences) were used as secondary antibodies. The signals were visualized with a VECTASTAIN Elite ABC Standard Kit (Vector Laboratories) and 3′,3′-diaminobenzidine (DAB; K3468, DAKO), and counterstained with hematoxylin.

Western blotting

Protein extraction from tumor tissues and cultured cells, and Western blotting were carried out as previously described (29). For the detection of midkine in medium, cultured cells in a 6-well dish were exposed to serum-free medium containing 20 μg/mL of heparin sodium salt. Twelve hours later, the media were harvested and the cells were washed with PBS. Midkine is easily attached to the cell surface or extracellular matrix. When we detect the secreted midkine level, we usually add heparin because midkine possesses strong affinity to heparin, and heparin-bound midkine is kept in the medium. For the detection of Notch2 (ICD), cultured cells and tumor tissues were suspended in low-salt buffer [10 mmol/L HEPES-KOH pH 7.8, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.25% NP-40, protease inhibitor cocktail, and phosphatase inhibitor cocktail (Nacalai Tesque)], and homogenized on ice with dounce homogenizer. After the centrifuge (4,000 rpm, 5 minutes), the supernatants were mixed with sample buffer (cytosol and membrane extract). The pellets were homogenized with low-salt buffer again, and sonicated in radioimmunoprecipitation assay (RIPA) buffer (nucleus extract). Protein amounts were quantified (BCA Protein Assay Kit, Thermo), and subjected to SDS-PAGE. The following primary antibodies were used: goat anti-human midkine (1:1,000; ref. 30), mouse anti-β-actin (A5441, 1:1,000; Sigma), rabbit anti-ALK (3333, 1:1,000; Cell Signaling), rabbit anti-phospho-ALK (3341, 1:1,000; Cell Signaling), rabbit anti-histone H3 (9715, 1:1,000; Cell Signaling). Antibody against Notch2 (ICD) was the same as that for immunohistochemistry (1:1,000).

Surface plasmon resonance assay

Surface plasmon resonance (SPR) assays were carried out as previously described using a BIAcore T100 instrument (GE Healthcare; ref. 23). The 3′-biotinylated Apt-1 and negative control RNA (Apt-nc) were immobilized on the flow cell 2 and 3 of a Sensor Chip SA (GE Healthcare), respectively. The resonance units (RU) of these immobilized oligonucleotides
were about 270. Human midkine (Peptide Institute Inc.), human PTN (Peptide Institute Inc.), human IgG1 (Calbiochem), and human albumin (Sigma) were dissolved in the running buffer with 0.5 g/L transfer RNA (Sigma). The final concentrations were 100 nmol/L. The running buffer was a mixture of 145 mmol/L NaCl, 5.4 mmol/L KCl, 1.8 mmol/L CaCl$_2$, 0.8 mmol/L MgCl$_2$, 20 mmol/L pH 7.6 Tris–HCl, and 0.05% Tween 20.

**Alkaline phosphatase assay**

The cDNA for human midkine coding the region from Lys23 to the stop codon was cloned into an APTag-5 vector using XhoI and XhoI sites. 293T cells were plated onto a 10-cm dish ($2.0 \times 10^6$ cells per dish). The next day, 293T cells were transfected with APTag-5 or APTag-5-midkine vector using FuGENE6 (Roche). Five days later, the media were harvested and filtered through a 0.22-μm polyethersulfone membrane.
The collected alkaline phosphatase proteins were kept at 4°C. For in vitro assay, TNB1 cells (0.1 × 10⁶ cells per well) were plated in 6-well dishes coated with collagen I. Twenty-four hours later, the cells were washed with HBHA buffer [ Hank’s balanced salt solution with 0.5 mg/mL bovine serum albumin, 20 mmol/L HEPES pH 7.0] and incubated with 1 U/mL of alkaline phosphatase or alkaline phosphatase–midkine and 100 nmol/L of each aptamer for 90 minutes at room temperature. Then the cells were washed 5 times with HBHA and lysed with 200 μL of cell lysis buffer (Q504, GenHunter). Endogenous alkaline phosphatase was inactivated at 65°C for 10 minutes. Fifty microliter of the cell lysate was mixed with an equal volume of alkaline phosphatase assay reagent A (Q501, GenHunter), and incubated at 37°C for 15 minutes. Hundred microliter of 0.5 mol/L NaOH was added to stop the reaction, and the OD₄₀₅ value was measured to calculate the alkaline phosphatase activity according to the instructions from GenHunter (31, 32).

Soft agar assay
A total of 1.5 mL of bottom agar (0.5% agar/RPMI + 10% FBS) was plated on 6-well dishes (n = 3). One milliliter of top agar (0.33% agar/RPMI + 10% FBS) containing TNB1 cells (2,000 cells per well), YT-nu cells (2,000 cells per well), or LNCap cells (5,000 cells per well) with or without 100 nmol/L of each aptamer and 100 ng/mL of recombinant human midkine (R&D, 258-MD) was plated on the bottom agar. On the next day, 1 mL of serum-free RPMI was added to each well. Two weeks later, the colonies were stained with crystal violet and counted (6 fields per well).

Therapeutic experiments using a xenograft model in nude mice
TNB1 cells (5 × 10⁶ cells in 50% Matrigel; BD Biosciences) or YT-nu cells (1 × 10⁶ cells in 50% Matrigel) were subcutaneously inoculated into the left and right flank of 8-week-old athymic nude mice (KSN/Sle; SLC, Japan). One week later, when the tumors reached a volume of approximately 50 to 80 mm³, the mice were randomly divided into groups (n = 4 each). A total of 100 μg/tumor of each aptamer was intratumorally administrated twice per week, and tumor volumes were calculated with the following formula: volume (mm³) = (width)² × length/2 (33).

Statistical analysis
Results are presented as the mean ± SD. Their homoscedasticities were checked by f test. Statistical significance was evaluated with a 2-tailed, unpaired t test. In Supplementary Fig. S8C, Welch correction was applied.

Results
Midkine is expressed in the precancerous lesions of MYCN transgenic mice
At 2 weeks of age, while the SMG in wild-type mice consisted of fully differentiated ganglion cells, undifferentiated neuroblasts (small round cells) were locally accumulated in the SMG of MYCN transgenic mice; these cells are collectively referred to as a hyperplasia (Fig. 1A, arrowheads). These observations were consistent with previous reports (20, 21, 29). Although the SMG in wild-type mice expressed a low level of midkine mRNA, the level was significantly increased in MYCN transgenic mice (Fig. 1B). Quantitative real-time PCR showed that midkine mRNA was upregulated approximately 5-fold in MYCN transgenic mice (Fig. 1C). Other midkine-related genes were also examined (Fig. 1B and Supplementary Fig. S1). Notch2 (34, 35), LRP1 (36–38), PTPζ (39), and integrins (α4, 6β, 1β; ref. 40) are candidates for midkine receptors, and they were similarly expressed in both wild-type and MYCN transgenic mice. ALK is another receptor candidate (41). Interestingly, ALK mRNA was dominantly expressed in MYCN transgenic mice (Fig. 1B). PTN is the only family member of midkine. In contrast to midkine, the PTN expressions were constant between wild-type and MYCN transgenic mice (Fig. 1B).

Immunohistochemical staining also revealed that the midkine protein was intensively expressed in hyperplasia lesions (Fig. 1D). Neuroblasts in hyperplasia lesions strongly expressed the transgene MYCN and the proliferation marker Ki-67 and were negative for the differentiation marker tyrosine hydroxylase (Fig. 1E), as previously reported (20, 42). Taken together, these results showed that midkine was highly expressed in precancerous hyper-proliferative cells. Furthermore, midkine and the related genes were continuously expressed in the growing tumor (Supplementary Fig. S2).
The suppression of tumor incidence and progression in midkine-knockout mice

Next, we addressed the involvement of endogenous midkine for the tumorigenesis of MYCN transgenic mice. To this end, we crossed the midkine-knockout mice with MYCN transgenic mice, and monitored their phenotype. As shown in Fig. 2A, MYCN transgenic mice (hemizygotes) began to die from 6 weeks of age, and the peak of the deaths from tumor occurred at an age of 9 to 14 weeks. As a result, around 30% of MYCN transgenic mice were free of tumor. On the other hand, MYCN transgenic mice possessing homozygous deletion of the midkine gene (midkine−/−/−) exhibited the suppressed phenotype (Fig. 2A). Their tumor deaths were delayed for 1 to 2 weeks and more than 40% of them were tumor-free. Midkine+/−/− mice expressed significantly lower levels of midkine proteins compared with midkine+/−/+ mice but still expressed some (Fig. 2B). The survival curve of midkine+/−/− mice was intermediate between those of midkine+/−/+ and midkine−/−/− mice. Taken together, our data suggested that midkine is involved in the tumor incidence and progression of MYCN transgenic mice.

Attenuation of Notch2 activity in the precancerous lesions of midkine-deficient mice

Recently, midkine-Notch2 signaling in pancreatic cancer was reported (35). Because midkine-knockout mice showed a tumor-suppressive phenotype (Fig. 2A), we examined whether Notch2 activity was attenuated in these mice. The full-length and the ICD of Notch2, an active form that translocates into the nucleus, were immunostained to evaluate the activation of Notch2 in the SMG of 2-week-old precancerous mice. Midkine+/−/− mice showed both full-length and ICD staining at the hyperplasia lesion (Fig. 3A and B), which indicates the activation of Notch2 signaling. In contrast, although full-length was also expressed in midkine−/−/− mice (Fig. 3D), ICD staining was markedly diminished compared with midkine+/−/− mice (Fig. 3E). In addition, the expression of HES1, one of the major target genes of Notch, was also decreased in midkine−/−/− mice (Fig. 3C and F). These results indicate the attenuation of the Notch2 signal in midkine−/−/− mice, and strongly suggest the possibility that midkine signals via Notch2 to regulate early tumorigenesis in MYCN transgenic mice.

An RNA aptamer targeting midkine inhibits its binding to the cell surface

For the purpose of targeting midkine protein, we selected RNA aptamers to midkine from a complex library of random RNA sequences through the SELEX process. The initial isolates of anti-midkine aptamers contained several variants of distinct properties. Of these, we isolated the midkine-specific aptamer designated as Apt-1. In addition, we synthesized a scrambled

![Image](https://cancerres.aacrjournals.org/content/73/4/1322/F3.large.jpg)

**Figure 3.** Activation of Notch2 is diminished in the precancerous lesions of midkine−/− mice. The 2-week-old SMG from midkine−/− (A–C) or midkine−/− (D–F) MYCN transgenic (hemizygous) mice was subjected to immunohistochemistry with the full-length (FL; A and D) or the ICD (B and E) of Notch2 and HES1 (C and F). Bottom, magnifications of the boxed region in top. Notch2 (ICD), an activated form, and HES1 were markedly diminished in midkine−/− mice (E and F) compared with hemizygous midkine+/− mice (B and C). Scale bar, 200 μm.
negative control. Apt-nc. SPR analysis indicated that Apt-1 specifically recognized midkine but not PTN and other plasma proteins. For the SPR assays, each RNA aptamer was bound to the surface of the sensor chip and 100 nmol/L of each protein (midkine, PTN, human IgG1 [hIgG1], and human serum albumin [HSA]) was injected. B, preparation of alkaline phosphatase–midkine fusion protein. The media of 293T cells transfected with expression vectors were collected and used for the Western blotting with anti-alkaline phosphatase or anti-midkine antibodies. C, in vitro alkaline phosphatase assay. TNB1 cells were treated with the alkaline phosphatase–midkine fusion protein in the presence of each aptamer, and the alkaline phosphatase activity bound on the cell surface was measured. *, P < 0.001. D, TNB1 cells were treated with 100 nmol/L of aptamers for 24 hours, and Notch2 (ICD) and histone H3 (internal control) were detected by Western blotting. E, the expression of HES1 mRNA was examined by real-time PCR using samples with the same condition as D. *, P < 0.05.

Figure 4. An RNA aptamer targeting midkine inhibits its binding to the cell surface. A, SPR assays showed that the RNA aptamer Apt-1 bound to midkine but not to PTN and other plasma proteins. For the SPR assays, each RNA aptamer was bound to the surface of the sensor chip and 100 nmol/L of each protein [midkine, PTN, human IgG1 (hIgG1), and human serum albumin (HSA)] was injected. B, preparation of alkaline phosphatase–midkine fusion protein. The media of 293T cells transfected with expression vectors were collected and used for the Western blotting with anti-alkaline phosphatase or anti-midkine antibodies. C, in vitro alkaline phosphatase assay. TNB1 cells were treated with the alkaline phosphatase–midkine fusion protein in the presence of each aptamer, and the alkaline phosphatase activity bound on the cell surface was measured. *, P < 0.001. D, TNB1 cells were treated with 100 nmol/L of aptamers for 24 hours, and Notch2 (ICD) and histone H3 (internal control) were detected by Western blotting. E, the expression of HES1 mRNA was examined by real-time PCR using samples with the same condition as D. *, P < 0.05.
showed no effect (Fig. 4C and Supplementary Fig. S4). These results indicate that Apt-1 exerts a specific and effective activity to block midkine binding to the cell surface.

**The RNA aptamer Apt-1 suppressed the growth of neuroblastoma cells both in vitro and in vivo**

Consistent with its activity to block the binding of midkine to the cell surface, Apt-1 treatment slightly suppressed the production of Notch2 (ICD; Fig. 4D), and significantly reduced the expression of HES1 mRNA in TNB1 cells (Fig. 4E). Next, we evaluated the anchorage-independent colony formation of TNB1 cells. TNB1 cells were cultured in soft agar plates in the absence or presence of RNA aptamers. As a result, the addition of Apt-1 effectively blocked the colony formation in soft agar (Fig. 5A and Supplementary Fig. S5). In contrast, Apt-nc showed no inhibitory effects. The same results were observed in another neuroblastoma cell line, YT-nu (Supplementary Fig. S7A). Importantly, the addition of recombinant midkine protein could cancel the inhibitory effect of Apt-1 (Fig. 5A, *P < 0.001). B, soft agar assay with LNCap cells. Apt-1 could not suppress the colony formation of LNCap cells. C, Western blotting to detect secreted midkine from each cell line. LNCap cells showed little midkine expression. D, the experimental scheme for the RNA aptamer-mediated midkine-targeting therapy with TNB1 xenograft tumors. E, tumor growth curves (volume). *P < 0.01. F, tumor weights at 3 weeks. *P < 0.01.

**Notch2 signaling was suppressed in Apt-1–treated tumors**

To confirm the effect of Apt-1, we investigated the Notch2 signaling in Apt-1–treated xenograft tumors. Immunohistochemistry (Fig. 6A) and Western blotting (Fig. 6B) showed that Notch2 (ICD) levels were suppressed in Apt-1–treated tumors. In addition, the expression of HES1 protein (Fig. 6A) and mRNA (Fig. 6C) were significantly decreased in Apt-1–treated tumors. In contrast, Apt-1 exerted no obvious inhibitory effect on ALK activation (Fig. 6B). We confirmed the same results with YT-nu cells (Supplementary Fig. S8). Taken together, these results suggest that the antitumor effect of Apt-1 should be mediated by the inhibition of midkine-Notch2 axis.
Discussion

Here, we have shown that endogenous midkine is involved in the tumorigenesis of neuroblastoma in MYCN transgenic mice. At the age of 2 weeks, MYCN transgenic mice showed the hyperplasia phenotype in the SMG, in which undifferentiated neuroblasts were locally accumulated (Fig. 1A). Although all MYCN transgenic mice at 2 weeks of age showed the hyperplasia status, only around 70% of them developed neuroblastoma afterward (Fig. 2A). These results indicate that the SMG at 2 weeks of age would not be completely tumorigenic. We found that midkine was more highly expressed at those hyperplasia lesions than in the surrounding differentiated ganglion cells (Fig. 1B–D). Furthermore, midkine deficiency suppressed the tumor incidence and growth rate in MYCN transgenic mice (Fig. 2), which supports the idea that midkine is involved in the tumorigenesis of neuroblastoma. PTN is the only family member of midkine. In terms of the relationship between their expression levels and prognoses, midkine and PTN show opposite profiles. That is, high midkine and low PTN expressions are related to poor prognosis (18). In contrast to midkine, PTN was equally expressed in the 2-week SMG of MYCN transgenic and wild-type mice (Fig. 1B). Therefore, despite the structural similarity, PTN might not be involved in the tumorigenesis of neuroblastoma in MYCN transgenic mice.

The tumor incidence observed in MYCN transgenic mice was suppressed in midkine-deficient mice (Fig. 2A). Moreover, Apt-1-mediated targeting of midkine in xenograft model revealed an outstanding tumor-suppressive effect (Fig. 5 and Supplementary Figs. S6 and S7). But, there is a difference between midkine-deficient mice and xenograft model in terms of the effectiveness of midkine targeting.

We used the RNA aptamer to target midkine. It is important to note that RNA aptamers are considered to be highly potential therapeutics. Indeed, anti-VEGF aptamer, Macugen, is in market for curing age-related macular degeneration, and other 9 programs are now in clinical studies (43, 44). The SPR study showed that Apt-1 could bind to midkine much more strongly than PTN and other plasma proteins (Fig. 4A and Supplementary Fig. S3), suggesting that Apt-1 could specifically bind to midkine in physiologic conditions. Intratumor administration of Apt-1 dramatically suppressed the growth of a subcutaneous tumor derived from TNB1 (Fig. 5) or YT-nu cells (Supplementary Fig. S7). Because RNA aptamers chemically modified to improve their pharmacokinetics and can be applied for systemic administration (26), they could be efficient tools to target midkine.
midkine in patients with neuroblastoma with an unfavorable prognosis.

So far, the intracellular signaling pathway activated by midkine has not been fully elucidated. Among the candidates for its receptor, ALK has a close relationship to the pathogenesis of neuroblastoma. Thus, ALK has been shown to be the predominant position gene for familial neuroblastoma, and it is also somatically mutated and hyperactivated in some patients with neuroblastoma (45–48). Interestingly, ALK is the only receptor candidate showing the dominant expression in the precancerous SMG of MYCN transgenic mice (Fig. 1B and Supplementary Fig. S1). This might suggest that midkine and ALK function cooperatively during the tumorigenesis of neuroblastoma. However, we also found that Apt-1 could efficiently suppress the growth of xenograft tumor derived from TNB1 cells (Fig. 5). TNB1 cells possess the R1275Q mutation, one of the most common hyperactive mutations in the ALK gene. Our data suggest that midkine functions independently of ALK in terms of the regulation of tumorigenesis. Consistent with this, the knockdown of midkine with short hairpin RNA (shRNA) can suppress the growth of SH-SY5Y cells, an neuroblastoma cell line possessing another hyperactive mutation (F1174L) in the ALK gene (data not shown).

On the basis of these observations, we speculate that a receptor other than ALK is vital for midkine signaling in the tumorigenesis of neuroblastoma. In this context, our finding on Notch2 is interesting. Recently, it has been reported that Notch2 acts as a functional receptor of midkine in pancreatic cancer, and midkine-Notch2 signaling regulates the epithelial–mesenchymal transition and chemotherapy resistance (35). Notch signaling is involved in the early development of the neural crest (49). The tumorigenesis of neuroblastoma and the development of neural crest-derived cells may share some molecular mechanisms. Here, we found that midkine deficiency resulted in attenuated Notch2 activation in the hyperplasia lesions of the SMG in 2-week-old MYCN transgenic mice (Fig. 3). This is the first physiologic data in vivo suggesting the involvement of midkine in a particular type of receptor signaling. Recently, it has been reported that midkine and ALK were cooperatively involved in the normal development of chicken sympathetic neurons (50). Our current results indicate that Notch2 should function as a midkine receptor during the tumorigenesis of neuroblastoma. However, these 2 studies do not exclude the possibility that there is a functional relationship between the midkine–ALK and midkine–Notch2 pathways. This issue should be verified in the future study. Finally, to unveil the molecular mechanism by which midkine–Notch2 signaling regulates the pathogenesis of neuroblastoma, the identification of target genes other than HES1 should be a future topic of research.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: S. Kishida, A. Onishi, Y. Nakamura, K. Kadomatsu Development of methodology: S. Kishida, P. Mu, S. Miyakawa, K. Sakamoto Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Kishida, S. Miyakawa, K. Kadomatsu, P. Mu Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Kishida, P. Mu, S. Miyakawa Writing, review, and/or revision of the manuscript: S. Kishida, S. Miyakawa, A. Onishi, Y. Nakamura, K. Kadomatsu Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Miyakawa, K. Kadomatsu Study supervision: M. Fujisawa, Y. Nakamura, K. Kadomatsu

Acknowledgments

The authors thank Drs. Makoto Sawada and Kenji Ono, Department of Brain Functions, Division of Stress Adaptation and Protection, Research Institute of Environmental Medicine, Nagoya University, for the use of MRI equipment. The authors also thank Misako Tanase for her technical assistance and members of RIBOMIC Inc. for discussion.

Grant Support

This work was supported in part by a Grant-in-Aid for Cancer Research (20-13) from the Ministry of Health, Labour and Welfare (MHLW), Japan to K. Kadomatsu, a Grant-in-Aid from the National Cancer Center Research and Development Fund (22-2) to K. Kadomatsu, Grants-in-Aid (23119002 and 23390078) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan to K. Kadomatsu, Grants-in-Aid (22790311 and 24590777) from MEXT to S. Kishida, a Grant-in-Aid from the Ministry of Agriculture, Forestry and Fisheries, Japan to K. Kadomatsu and A. Onishi, the funds from the Global COE program of MEXT, Japan to Nagoya University, Core Research for Evolution Science and Technology (CREST) grant from the Japan Science and Technology Agency to Y. Nakamura, and research grants to Y. Nakamura from MEXT and MHLW.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 3, 2012; revised November 19, 2012; accepted December 3, 2012; published OnlineFirst December 14, 2012.

References


