BJP British Journal of Pharmacology

DOI:10.1111/bph.12442
www.brjpharmacol.org

Themed Section: Midkine

REVIEW

Involvement of midkine in neuroblastoma tumourigenesis

S Kishida and K Kadomatsu

Department of Biochemistry, Nagoya University Graduate School of Medicine, Nagoya, Japan

Correspondence
Kenji Kadomatsu, Department of Biochemistry, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan. E-mail: kkadoma@med.nagoya-u.ac.jp

Keywords
neuroblastoma; RNA aptamer; Notch2; ALK; chemoresistance

Received
3 July 2013
Revised
9 September 2013
Accepted
16 September 2013

Midkine is highly expressed in various cancers, including neuroblastoma, one of the most malignant paediatric solid tumours known. Also, it has been shown to be useful as a tumour marker, a prognosis factor and a target of molecular therapy. Several molecular tools (e.g. siRNA, antibodies and RNA aptamer) have been used to establish a midkine-targeted therapy. The involvement of midkine in tumourigenesis has been demonstrated *in vivo* in a mouse neuroblastoma model, where targeting it with an RNA aptamer was shown to be an effective treatment for xenografted tumours. Chemoresistance is one of the notable phenotypes regulated by midkine in various cancer cell types. In pancreatic tumours and glioma cells, midkine is expressed in chemoresistant cells and is involved in the survival of these cells in the presence of anticancer drugs. In contrast to these tumours, midkine was found to be expressed in every neuroblastoma cell line tested and the knockdown of midkine alone was sufficient to suppress their growth. These results indicate that neuroblastoma cells are highly dependent on midkine and that a midkine-targeted therapy could exert a significant effect in these cells. However, to achieve a midkine-targeted therapy for high-risk neuroblastoma patients, the further refinement of the RNA aptamer or antibody as tools and the elucidation of midkine signalling are immediate issues that need to be resolved. Regarding the latter, although it has been shown that Notch2 functions as a receptor in neuroblastoma cells, it is likely that other receptors (e.g. anaplastic lymphoma kinase) are also involved in midkine signalling.

LINKED ARTICLES
This article is part of a themed section on Midkine. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2014.171.issue-4

Abbreviations
ALK, anaplastic lymphoma kinase; EMT, epithelial-mesenchymal transition; HES1, hairy and enhancer of split 1; LRP1, LDL receptor-related protein 1; PDAC, pancreatic ductal adenocarcinoma; SG, sympathetic ganglion; SMG, superior mesenteric ganglion; Tg, transgenic; TH, tyrosine hydroxylase

Midkine in various cancers

Midkine is a heparin-binding growth factor identified over 20 years ago (Kadomatsu *et al.*, 1988; Tomomura *et al.*, 1990). Midkine was discovered through the screening of differentially expressed genes during the differentiation of embryonal carcinoma cells (Kadomatsu *et al.*, 1988). Midkine protein, whose molecular weight is 13 kDa, is rich in basic amino acids and cysteines, and shares around 50% sequence identity with another heparin-binding growth factor, pleiotrophin (Kadomatsu *et al.*, 1988; Tomomura *et al.*, 1990). These two compounds comprise the entire midkine family of growth factors. The midkine protein is composed of two domains, the N- and C-terminal half domains respectively. Each domain contains three anti-parallel β sheets that are linked by a flexible linker region. Interestingly, only the C-terminal-half domain of midkine exhibits biological activities, for example neurite outgrowth, fibrinolysis and nerve cell migration.

So far, it has been shown that midkine is involved in a huge variety of biological phenomena, including development, inflammation, tissue protection and blood pressure (Kadomatsu and Muramatsu, 2004; Kadomatsu *et al.*, 2013). For example, ectopically expressed midkine can transform NIH3T3 cells *in vitro* (Kadomatsu *et al.*, 1997). One of the most notable characteristics of midkine is its significant expression in a variety of cancers. It has been shown to be highly expressed in Wilms' tumour (Tsutsui *et al.*, 1993), gastrointestinal cancer (Aridome *et al.*, 1995), astrocytoma (Mishima *et al.*, 1997), colorectal cancer (Ye *et al.*, 1999), prostate cancer (Konishi *et al.*, 1999) and neuroblastoma (Nakagawara *et al.*, 1995). In contrast, in healthy animals its

Table 1
Cancer therapies targeting midkine

Tools	Experiments	References
Oligodeoxynucleotide	Intratumour injection to CMT-93 (mouse rectal carcinoma) xenograft	Takei *et al.*, 2001 Takei *et al.*, 2002
Morpholino oligomer	Intratumour injection to PC-3 (prostate carcinoma) and SW620 (colon carcinoma) xenografts	Takei *et al.*, 2005
siRNA	Intratumour injection to PC-3 xenograft	Takei *et al.*, 2006
Oligonucleotide	I.v. injection to *in situ* hepatocellular carcinoma model	Dai *et al.*, 2007a
Oligonucleotide	I.v. injection to *in situ* hepatocellular carcinoma model	Dai *et al.*, 2007b
MK-TRAP (midkine-binding peptide derived from LRP1)	MK-TRAP-transfected CMT-93 xenograft	Chen *et al.*, 2007
Polyclonal antibody	Anchorage-independent colony formation of G401 (Wilms' tumour) and CMT-93	Chen *et al.*, 2007
Oligonucleotide-loaded nanoparticle	I.v. injection to *in situ* hepatocellular carcinoma model	Dai *et al.*, 2009
siRNA	Intratumour injection to T98 (glioma) xenograft	Lorente *et al.*, 2011
Monoclonal antibody	I.p. injection to 143B (osteosarcoma) xenograft	Sueyoshi *et al.*, 2012
RNA aptamer	Intratumour injection to TNB1 and YT-nu (neuroblastomas)	Kishida *et al.*, 2013

expression is mostly restricted to midgestation during embryogenesis (Kadomatsu *et al.*, 1990). These findings strongly suggest that midkine would be useful not only as a tumour marker but also as a molecular target for cancer therapy. And indeed, serum midkine levels have been shown to be a reliable tumour marker, as well as a prognostic factor, in several cancers, including neuroblastoma (Ikematsu *et al.*, 2000; 2003; 2008; Obata *et al.*, 2005; Jia *et al.*, 2007; Ota *et al.*, 2008; Ibusuki *et al.*, 2009). In cases of neuroblastoma, the plasma midkine level is significantly correlated with known prognostic factors, such as *MYCN* amplification, low *TRKA* expression, diploidy, stage 3 and 4 disease, and over 18 months old at diagnosis. In addition, there is a striking correlation between a high plasma midkine level itself and poor prognosis (Ikematsu *et al.*, 2003; 2008).

With regard to the use of midkine as a molecular target in cancer therapy, several molecular tools have been established to target midkine, and have been shown to exert therapeutic effects on xenografted tumours derived from several types of cancer (Table 1). These tools can be mainly classified into three groups: (i) RNAi-based nucleotides (Takei *et al.*, 2001; 2002; 2005; 2006; Dai *et al.*, 2007a,b; 2009; Lorente *et al.*, 2011); (ii) small peptides or antibodies (Chen *et al.*, 2007; Sueyoshi *et al.*, 2012); and (iii) RNA aptamers (Kishida *et al.*, 2013). They are expounded in the next section.

Involvement of midkine in neuroblastoma

Although it has been reported that midkine is highly expressed in a variety of cancers (Tsutsui *et al.*, 1993; Aridome *et al.*, 1995; Nakagawara *et al.*, 1995; Mishima *et al.*, 1997; Konishi *et al.*, 1999; Ye *et al.*, 1999) and that its suppression results in growth inhibition of tumour cells (Takei *et al.*, 2001; 2002; 2005; 2006; Chen *et al.*, 2007; Dai *et al.*, 2007a,b; 2009; Lorente *et al.*, 2011; Sueyoshi *et al.*, 2012; Kishida *et al.*, 2013), no direct or conclusive evidence shows that midkine is involved in tumourigenesis. In order to address this point, *in vivo* data utilizing animal models is needed. Recently, the involvement of midkine in neuroblastoma, one of the most malignant paediatric solid tumours known, was revealed for the first time in a study using a mouse model (Kishida *et al.*, 2013).

Neuroblastoma develops from a neural crest-derived sympathetic neuronal lineage, and accounts for around 15% of all paediatric cancer deaths (Nakagawara and Ohira, 2004). Its prognosis still remains poor despite an enormous amount of basic and clinical research. However, neuroblastoma is also famous for the unique characteristics of a subclass of tumours classified as stage 4S ('S' stands for 'special'). The tumours in patients with stage 4S disease (with metastases in the liver, skin or bone marrow) subsequently disappear through apoptosis and/or differentiation without any treatment. In other words, stage 4S patients can be free from malignancy provided they can withstand the life-threatening period when the tumours are large. This phenomenon is called spontaneous regression, and its molecular mechanisms are almost entirely unknown. It is very important for us to investigate and understand the mechanism of spontaneous regression, since the phenomenon could provide clues to the development of novel therapies for malignant neuroblastomas other than stage 4S. Midkine mRNA is highly expressed in neuroblastoma tissues with poor prognosis, whereas pleiotrophin, another family member of midkine, is expressed in tumours with good prognosis (Nakagawara *et al.*, 1995). Although these two proteins seem to have redundant functions in some fields, this does not appear to be the case with neuroblastoma. As mentioned above, increased serum midkine levels appear to be associated with poor prognostic factors in human neuroblastoma (Ikematsu *et al.*, 2000; 2003; 2008).

The oncogenic transcription factor *MYCN*, which is a member of the same family as *c-Myc*, is considered to be the most important gene indicative of a predisposition for the development of neuroblastoma, and its amplification is one of the most important prognosis factors so far. The *MYCN* gene initially plays a role in the normal development of sympathetic neurons (Nakagawara and Ohira, 2004), but the ectopic expression of *MYCN* at a particular stage of development is thought to be a trigger for oncogenesis, and as a result, the neuroblasts in which human *MYCN* is ectopically expressed remain in an undifferentiated state and proliferate *ad infinitum*. *MYCN* transgenic (Tg) mice in which the human *MYCN* gene was introduced under the control of a rat tyrosine hydroxylase (TH) promoter have been developed as an animal model for neuroblastoma, (Weiss *et al.*, 1997). TH is a sympathetic neuron-specific enzyme, and its promoter is turned on in neural crest-derived migrating cells whose lineages are committed to sympathetic neurons during early development. *MYCN* Tg mice spontaneously develop tumours from the superior mesenteric ganglion (SMG), one of the sympathetic ganglia. The SMG lies on the superior mesenteric artery between the left and right kidneys, and its size is less than 1 mm in diameter. Although the most frequent origin of human neuroblastoma is the adrenal grand, the dominant origin in *MYCN* Tg mice is the SMG. These tumours are pathologically similar to human neuroblastoma with respect to both histology (Weiss *et al.*, 1997) and pattern of chromosomal aberration (Weiss *et al.*, 2000) and they have been utilized for a variety of basic investigations of neuroblastoma. At the age of 2 weeks, the SMG in wild-type mice consists of fully differentiated sympathetic ganglion cells, whereas in all *MYCN* Tg mice undifferentiated neuroblasts are accumulated in the SMG (Hansford *et al.*, 2004; Asano *et al.*, 2010; Huang *et al.*, 2011). These cells are collectively referred to as a hyperplasia. The hyperplasia might be a kind of precancerous stage, because most hyperplasia develop into advanced tumours. In *MYCN* Tg mice, midkine was highly expressed in both those precancerous SMG and later terminal tumour tissues (Kishida *et al.*, 2013). Next, in order to directly determine the involvement of midkine in the tumourigenesis of *MYCN* Tg mice, *Mdk* (the mouse gene name of midkine)-knockout mice were crossed with *MYCN* Tg mice. As a result, it was found that the genetic ablation of *Mdk* resulted in suppressed tumourigenesis (lower tumour incidence and delayed growth) of *MYCN* Tg mice (Kishida *et al.*, 2013). This is the first study to investigate the involvement of midkine in tumourigenesis at the *in vivo* level, and the results demonstrate that midkine has certain properties that promote the tumourigenesis of neuroblastoma.

It should be noted that the mechanism of action of midkine has not been fully elucidated. In terms of the initiation of midkine signalling, several receptor candidates, including anaplastic lymphoma kinase (ALK; Stoica *et al.*, 2002), Notch2 (Huang *et al.*, 2008; Güngör *et al.*, 2011; Kishida *et al.*, 2013), LDL receptor-related protein 1 (LRP1; Muramatsu, 2000; Chen *et al.*, 2007; Sakamoto *et al.*, 2011), receptor protein tyrosine phosphatase (PTPR) Z1 (Maeda *et al.*, 1999) and integrins (Muramatsu *et al.*, 2004), have been identified, but there is little solid evidence indicating the involvement of a particular receptor in the signalling pathway downstream of midkine. Recently, it was reported that the activated form of Notch2 (cleaved intracellular domain of Notch2 in nucleus) was significantly decreased in the precancerous lesions of *Mdk*-knockout *MYCN* Tg mice (Kishida *et al.*, 2013). Consistent with this, the expression of hairy and enhancer of split 1 (*Hes1*), a major target gene of Notch family members including Notch2, was also diminished (Kishida *et al.*, 2013). Taken together, these *in vivo* results suggest that a midkine-Notch2-HES1 signalling pathway is involved in neuroblastoma. The same pathway was previously implicated in pancreatic ductal adenocarcinoma (PDAC) (Güngör *et al.*, 2011). According to that report, the exposure of some PDAC cell lines to gemcitabine, the front-line chemotherapy used in PDAC treatment, induced both epithelial-mesenchymal transition (EMT) and a chemoresistant phenotype. It is not clear whether these two phenotypes are correlated. Although it was shown that the midkine-Notch2 pathway is involved in chemoresistance to gemcitabine, as yet, no evidence has been obtained *in vivo*.

Targeting midkine in neuroblastoma therapy

As listed in Table 1, several tools have been utilized to target midkine in cancer therapy. Intratumour injection of RNAi-based nucleotides [oligodeoxynucleotide (Takei *et al.*, 2001; Takei *et al.*, 2002), morpholino oligomer (Takei *et al.*, 2005) and siRNA (Takei *et al.*, 2006)] effectively suppressed the growth of xenografted tumours derived from CMT-93 cells (mouse rectal carcinoma) (Takei *et al.*, 2001; Takei *et al.*, 2002) and PC-3 cells (prostate carcinoma) (Takei *et al.*, 2005; Takei *et al.*, 2006). Intratumour injection of siRNA together with an antitumour drug also showed therapeutic effects (Lorente *et al.*, 2011). Furthermore, i.v. injections of these oligonucleotides into an *in situ* model of human hepatocellular carcinoma in mice suppressed the tumours to some extent (Dai *et al.*, 2007a,b; 2009). Also, the midkine-binding peptide has been utilized to neutralize the secreted midkine protein (Chen *et al.*, 2007). This peptide, designated as MK-TRAP, was derived from LRP1, one of the midkine receptors. MK-TRAP consists of 169 amino acids corresponding to a part of the huge extracellular domain of LRP1, and was revealed to have a strong affinity for midkine. MK-TRAP specifically interacted with midkine, and its expression in G401 (Wilms' tumour) cells resulted in growth inhibition in the monolayer culture. In addition, not only MK-TRAP expression but also the addition of MK-TRAP-containing medium suppressed the anchorage-independent colony formation of G401 and CMT-93 cells. Furthermore, CMT-93 cell-derived xenograft tumours that express MK-TRAP have been shown to exhibit suppressed growth *in vivo*. In the same study, polyclonal anti-midkine antibody was also shown to exert suppressive effects on anchorage-independent colony formation with CMT-93 and G401 cells (Chen *et al.*, 2007). Although therapeutic experiments with MK-TRAP and polyclonal antibody have not yet been done, these appear be potent tools for targeting midkine. With regard to antibodies, a recent report indicated that the growth of xenografted 143B cells (osteosarcoma) was inhibited by an i.p. injection of monoclonal antibody (Sueyoshi *et al.*, 2012). Although this *in vivo* effect

was not outstanding, the fact that systemic administration of this monoclonal antibody evoked a slight but clear effect suggests that it has potential for clinical application in the future. However, the use of an effective monoclonal antibody against midkine for neuroblastoma therapy will have to await further studies.

As described above, the involvement of midkine in the tumourigenesis of in *MYCN* Tg mice (Kishida *et al.*, 2013) strongly indicates that midkine-targeted therapy could provide an effective treatment for neuroblastoma. In the same report, an RNA aptamer against midkine notably showed a therapeutic effect in a xenografted tumour model (Kishida *et al.*, 2013). The RNA aptamer is the third candidate among the tools to target midkine (Table 1). RNA aptamers, which specifically recognize and directly bind to particular proteins, are considered nucleic acid analogues to antibodies. An RNA aptamer that specifically recognizes a particular molecule can be identified by screening from a complex library of random RNA sequences (20–80-mer), of typically 10^{14} different molecules, through a process known as systematic evolution of ligands by exponential enrichment. They form a particular three-dimensional structure and recognize a specific target in the same way as antibodies (Miyakawa *et al.*, 2006; 2008; Ishiguro *et al.*, 2011). Intratumour injection of the RNA aptamer against midkine (100 μg per shot, two shots per week) into neuroblastoma cell-derived xenografted tumours has been shown to suppress their growth significantly (Kishida *et al.*, 2013). The volume and weight of the treated tumours were around a quarter of the values in control tumours (Kishida *et al.*, 2013). These therapeutic effects of RNA aptamers were superior to those of the other tools listed in Table 1. In addition, both the activation of Notch2 and the expression of its target gene *Hes1* were simultaneously attenuated in the RNA aptamer-treated tumours (Kishida *et al.*, 2013). These results indicate that midkine-targeted therapy can be effective against neuroblastoma and also the suitability of RNA aptamer as a tool. Furthermore, the finding that the RNA aptamer treatment suppressed both the tumour growth and the Notct2-HES1 signalling pathway supports the hypothesis that the midkine-Notch2-HES1 pathway promotes the tumourigenesis of neuroblastoma *in vivo*. However, in contrast, it was reported that the expression level of *Hes1* in neuroblastoma cell lines was quite low compared with that in T-cell acute lymphoblastic leukaemia cell lines in which canonical Notch signalling was highly activated (Zage *et al.*, 2011). Although the expression of *Hes1* is decreased concomitantly with the inactivation of Notch2 in RNA-aptamer-treated tumour cells, the significance of this change has not yet been investigated (Kishida *et al.*, 2013). It is also possible that the canonical Delta/Jagged-Notch signal mainly induces *Hes1* as a target gene, and the midkine-Notch2 signal activates the expression of other genes involved in tumourigenesis (Figure 3). This possibility should be investigated further.

On considering all the tools for targeting midkine (Table 1), the use of an RNA aptamer might be slightly preferable in terms of effectiveness, relatively low immune stimulant activity, good productivity and applicability of modifications. However, further development of much more effective monoclonal antibodies is also a promising approach that should be pursued. With regard to siRNA and shRNA, a method to accurately and efficiently deliver these to tumour cells is a big problem that needs to be solved.

Induction of chemoresistance as a function of midkine

Although it has been suggested that the midkine-Notch2-HES1 signalling pathway promotes the tumourigenesis of neuroblastoma, the final output of midkine signalling has yet to be revealed not only in neuroblastoma but also in other cancers. Recently, several reports suggested that the induction of chemoresistance was one of the functions of midkine in some cancers. Firstly, as already mentioned, midkine was shown to confer gemcitabine resistance on PDAC cells (Güngör *et al.*, 2011). The chemoresistant PDAC cells induced midkine expression in response to gemcitabine treatment. In turn, midkine signalled via Notch2 to induce both EMT and chemoresistance against gemcitabine. Knockdown of either midkine induced by the drug or Notch2 resulted in the mesenchymal-epithelial transition and a chemosensitive phenotype. Whether MK-induced EMT can be correlated with chemoresistance has not yet been addressed. Secondly, midkine induced glioma cells to become resistant to the antitumour effects of cannabinoids (major active ingredient of marijuana) by signalling via ALK, one of the midkine receptors (Lorente *et al.*, 2011). There are cannabinoid-sensitive and -resistant glioma cell lines, and the latter express large amounts of midkine. The targeting of midkine induced the cannabinoid-sensitive phenotype to resistant cells, while the addition of midkine made cannabinoid-sensitive cells resistant *in vitro*. Midkine mediated this protective effect via ALK, and the midkine-ALK signal interfered with cannabinoid-induced autophagic cell death. Furthermore, siRNA-mediated silencing of midkine sensitized the xenografted chemoresistant cells to the antitumour effects of the cannabinoid *in vivo* (Lorente *et al.*, 2011) (Table 1). Other reports have also suggested that midkine induces chemoresistance in different cancers and to different drugs (Rebbaa *et al.*, 2001; Kang *et al.*, 2004; Mirkin *et al.*, 2005; Hu *et al.*, 2010; Xu *et al.*, 2012).

In contrast to the above results obtained in PDAC cells (Güngör *et al.*, 2011) and glioma cells (Lorente *et al.*, 2011), all tested neuroblastoma cell lines have been found to express a certain amount of midkine (Figure 1). This is probably because the neuroblastoma cell line was mainly established from patients with poor prognosis whose expression levels of midkine were expected to be high. As the neuroblastoma cells in patients with poor prognosis express high levels of midkine, it is likely they are chemoresistant. In fact, severe chemoresistance is one of the characteristics of relapsed neuroblastoma cells, and is clearly associated with patient mortality. Unexpectedly, in contrast to PDAC cells and glioma cells, the targeting of midkine alone was sufficient to suppress the growth of these cells under a monolayer culture condition (Figure 2). PDAC cells expressed midkine only in the presence of gemcitabine (Güngör *et al.*, 2011), and the depletion of midkine alone was not sufficient to kill chemoresistant glioma cells (Lorente *et al.*, 2011). In neuroblastoma cells, regardless of the status of the *MYCN* gene (amplified in

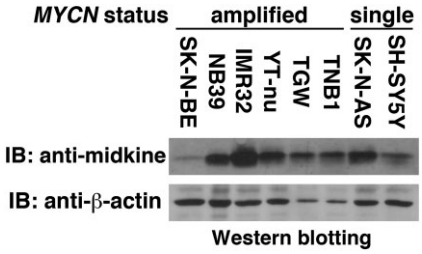

Figure 1

Midkine expression in neuroblastoma cell lines. Eight neuroblastoma cell lines were examined for the level of midkine secreted into medium by Western blotting. Each cell line in 6-well dishes was exposed to serum-free medium containing 20 μg·mL^{-1} of heparin sodium salt so that the heparin-bound midkine remains in the medium. Twelve hours later, the media containing heparin-bound midkine were harvested and, simultaneously, whole cell extracts were also prepared. β-actin in whole cell extracts was detected as a control for cell numbers. Six of the cell lines (SK-N-BE, NB39, IMR32, YT-nu, TGW, and TNB1) possessed an amplification of the *MYCN* gene, and two (SK-N-AS and SH-SY5Y) did not. Although the level of midkine expression in SK-N-BE cells was lower than that in the other cell lines, every cell line expressed a certain amount of midkine.

NB39 and SK-N-BE, single copy in SH-SY5Y) and the expression level of midkine (relatively low in SK-N-BE), the depletion of midkine caused significant growth inhibition in all cell lines (Figure 2). These results indicate the marked dependence of neuroblastoma cells on midkine, and highlight the strong potential of midkine-targeted neuroblastoma therapy. However, as there is still a possibility that midkine is also involved in chemoresistance in neuroblastoma cells, it would be worth evaluating the effect of combining midkine-targeted therapy with a low dose of chemotherapeutic agent (with little side effects) to achieve more effective therapeutic results. In fact, in a xenograft model of PC-3 cells, treatment with the combination of a sufficiently low dose of paclitaxel to avoid adverse effects and siRNA against midkine achieved a potent antitumour effect (Takei *et al.*, 2006). A combination therapy targeting midkine and an antitumour drug would thus be a promising approach to clinical treatment.

Midkine receptors in neuroblastoma cells

The results depicted in Figure 2 gave us additional information about the downstream signalling of midkine. As stated previously, there are several candidates for functional receptors of midkine (Maeda *et al.*, 1999; Muramatsu, 2000; Stoica *et al.*, 2002; Muramatsu *et al.*, 2004; Chen *et al.*, 2007; Huang *et al.*, 2008; Güngör *et al.*, 2011; Sakamoto *et al.*, 2011; Kishida *et al.*, 2013). Among them, Notch2 and ALK have so far been shown to be involved in neuroblastoma.

Originally the results from *in vitro* experiments suggested that ALK could function as a receptor of midkine (Stoica *et al.*, 2002). Furthermore, independently of its relationship with midkine, ALK has been shown to be a predisposition gene for familial neuroblastoma (Chen *et al.*, 2008; George *et al.*, 2008;

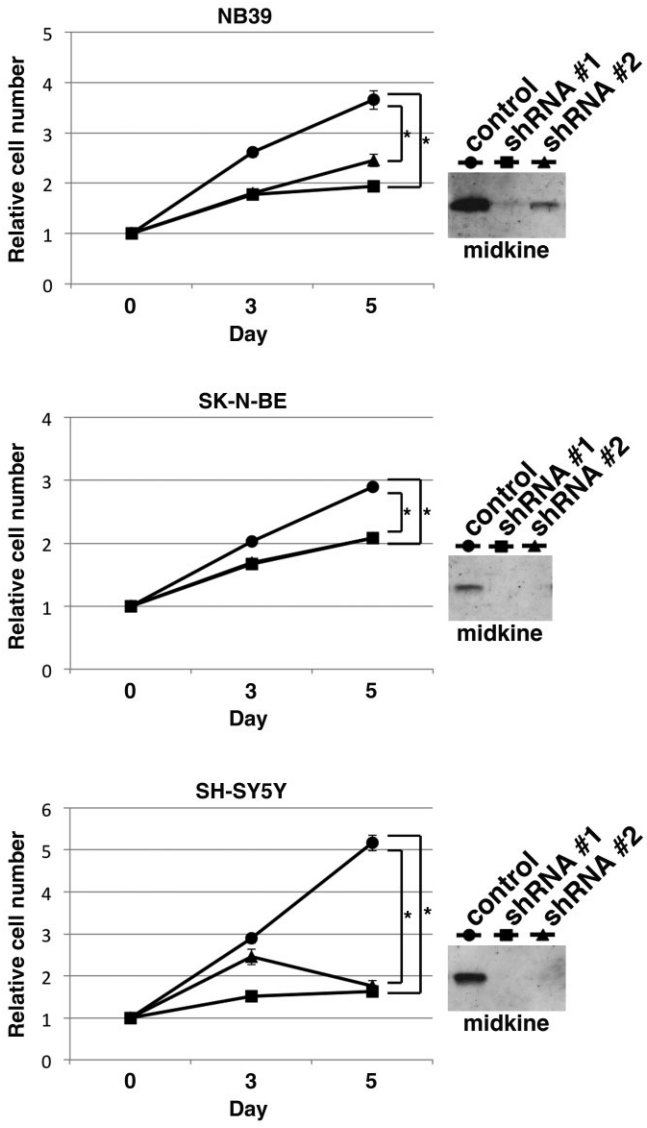

Figure 2

Knockdown of midkine suppressed the growth of neuroblastoma cell lines. Three neuroblastoma cell lines (NB39, SK-N-BE, and SH-SY5Y) were infected with lentivirus to express midkine-targeting shRNA [#1 (Sigma, St Louis, MO, USA: TRCN0000303918) and #2 (Sigma: TRCN0000331252)] or non-targeting shRNA (control). In order to confirm the knockdown efficiency, the levels of secreted midkine were examined by Western blotting (each right panel). Infected cells were plated in a 96-well dish (10 000 cells/well, 3% FBS), and assayed with a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) at 0, 3, 5 day respectively. *: $P < 0.001$ (unpaired t-test). *ALK* gene statuses in each cell line are as follows: amplified in NB39 cells, normal in SK-N-BE cells, and F1174L mutation in SH-SY5Y cells.

Janoueix-Lerosey *et al.*, 2008; Mossé *et al.*, 2008). Some hyperactive point mutations within its kinase domain (e.g. F1174L and R1275Q) have been identified in familial neuroblastoma, and they were also found in some sporadic cases (somatic mutations). In addition, the development of a mouse model of this disease revealed that those ALK mutations could be a trigger for oncogenesis of neuroblastoma (Berry *et al.*, 2012;

Heukamp *et al.*, 2012). However, the midkine-ALK axis has also been shown to be involved in the proliferation of normal sympathetic neurons in cultures of proliferating immature neurons from embryonic chick sympathetic ganglia (SG) (Reiff *et al.*, 2011). The *in vitro* proliferation of primary cultured embryonic chick SG was enhanced by the expression of ALK (F1174L), and was inhibited by an ALK inhibitor or the shRNA for ALK. Similarly, the shRNA for midkine was also able to inhibit the proliferation of these cells. Importantly, although treatment with the recombinant midkine protein successfully restored the proliferation of midkine shRNA-transfected SG, it showed no effect on ALK shRNA-transfected SG. Furthermore, the growth inhibition induced by midkine shRNA was restored by the expression of ALK (F1174L). These results suggest that the midkine-ALK axis regulates the proliferation of the normal SG during embryonic development. That the midkine-ALK axis probably functions in the normal development of sympathetic neurons is an important consideration when examining the molecular mechanism of the action of midkine in neuroblastomas. This is because the normal development of sympathetic neurons and the tumourigenesis of neuroblastomas could share some molecular mechanisms. The neural crest contains several lineages of cells differentiating into melanocytes, enteric neurons, sensory neurons and so on (Nakagawara and Ohira, 2004). Among them, only the cells differentiating into sympathetic neurons have the potential to proceed to neuroblastomas. This fact suggests that the mechanism by which neural crest cells differentiate into sympathetic neurons is essential for the tumourigenesis of neuroblastoma, and that a particular abnormal event during that process should turn the tumourigenic switch on. Taken together with the results from embryonic chick SG, which suggested the midkine-ALK axis is involved in the normal development of sympathetic neurons (Reiff *et al.*, 2011), these findings indicate that ALK functions as a receptor of midkine in neuroblastoma cells. Now, what do the results shown in Figure 2 imply? The *ALK* gene statuses in each cell line used in Figure 2 are as follows: amplified in NB39 cells, normal in SK-N-BE cells and F1174L mutation in SH-SY5Y cells. Hence, the results in Figure 2 indicate that the knockdown of midkine could suppress the growth of neuroblastoma cells regardless of the hyperactivated (amplification or hyperactive mutation) ALK statuses. These suggest that midkine has an ALK-independent function in neuroblastoma cells. Notch2 might mediate this ALK-independent midkine signalling (Figure 3). Of course, the possibility that midkine also signals through ALK in neuroblastoma cells cannot be ignored. The individual contributions of ALK, Notch2 and other receptors in neuroblastoma cells should be addressed in order to understand the actions of midkine.

Just as a reference, we would like to mention what is known about midkine and ALK orthologues. So far, there has been no physiological evidence to establish a ligand-receptor relationship between midkine and ALK. In order to address this point, knowledge based on the genetics of lower model animals (e.g. invertebrates) sometimes provides highly important information. That is, when a hierarchical ligand-receptor relationship is identified by the genetic study of lower model animals, the corresponding axis is frequently conserved in each mammalian orthologous gene. In terms of

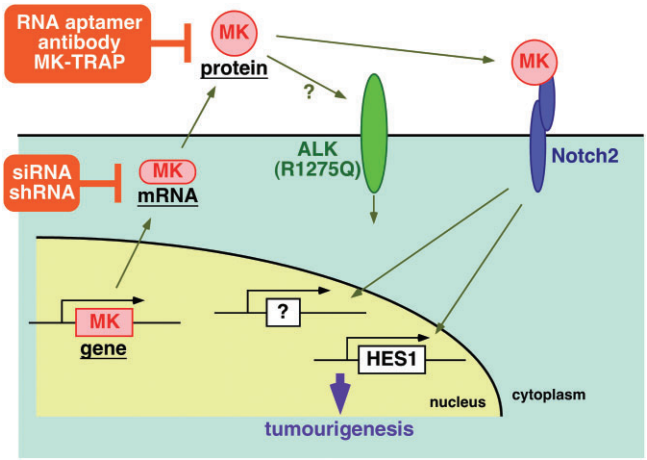

Figure 3

Midkine (MK) signalling in neuroblastoma cells. Neuroblastoma cells express and secrete midkine, which probably acts in an autocrine or paracrine manner. As shown here, siRNA and shRNA target midkine at the mRNA level to suppress its protein synthesis and secretion. In contrast, the RNA aptamer, antibody and MK-TRAP target the secreted midkine protein to suppress its binding to cell surface receptors. Notch2 was reported as a functional receptor of midkine in neuroblastoma cells. Although MK-Notch2 signalling induced the expression of *Hes1*, a canonical target gene of Notch family members, there might be other MK-Notch2-specific target genes involved in the tumourigenesis and tumour growth of neuroblastomas. Because an RNA aptamer against MK was effective in TNB1 cells that possess a hyperactive R1275Q mutation in ALK, ALK-independent MK signalling – that is, the MK-Notch2 axis – could function there. But there still might be a possibility that MK also signals via ALK in neuroblastoma cells.

midkine signalling, a study in *Drosophila* could be interesting. The orthologues of midkine and pleiotrophin are miple1 and miple2 (Englund *et al.*, 2006). The *Drosophila* orthologue of ALK has a proven ligand, named jelly belly (Jeb). Unfortunately, a mammalian orthologue of Jeb has not been identified. In addition, miple1 and miple2 show no homology to Jeb, and whether *Drosophila* ALK functions as a receptor of miple1 and miple2 has not been examined. Further progress in a *Drosophila* study would provide important information for our neuroblastoma research.

Future topics

Neuroblastoma is one of the most malignant tumours occurring in children. The current therapy consists of strong chemotherapy, radiation and surgery. Because there is almost no room to improve on these methods themselves, the introduction of a fourth method, a molecular-targeted therapy, is anticipated. Although midkine is a potent candidate for such therapy, there are several points that remain to be addressed. Firstly, the tools used to target midkine need to be refined. A significant therapeutic effect was recently achieved using intratumoural administration of an RNA aptamer (Kishida *et al.*, 2013). However, before this tool can be used clinically,

it will need to be further developed to allow for systemic administration. Because it has been suggested that some modifications could give aptamers enough stability for systemic administration (Ishiguro *et al.*, 2011), further progress is expected. Secondly, the details of midkine signalling in neuroblastoma cells remain to be clarified. Although a growing body of evidence indicates the involvement of midkine in various cancers, the molecular mechanism by which midkine signals are transmitted in cancer cells is not fully understood. This is partly because the receptors engaged in midkine signalling are themselves uncertain. Current data suggest that Notch2, instead of ALK, would act as a midkine receptor in neuroblastoma cells (Figure 3). But it is still possible that receptors other than Notch2 and ALK function in this fashion or that multiple receptors act cooperatively to transmit midkine signalling. Solving these problems to develop a robust therapy targeting midkine could prove greatly advantageous in the fight against neuroblastoma as well as in the treatment of various other midkine-expressing cancers.

Acknowledgements

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.K., a Grant-in-Aid from the National Cancer Center Research and Development Fund (22-4) to K.K., Grants-in-Aid (23110002, 23390078) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan to K.K., and Grants-in-Aid (22790311, 24590377) from MEXT to S.K.

Conflict of interest

The authors declare that they have no potential conflicts of interest to disclose.

References

Aridome K, Tsutsui J, Takao S, Kadomatsu K, Ozawa M, Aikou T *et al.* (1995). Increased midkine gene expression in human gastrointestinal cancers. Cancer Res 86: 655–661.

Asano Y, Kishida S, Mu P, Sakamoto K, Murohara T, Kadomatsu K (2010). DRR1 is expressed in the developing nervous system and downregulated during neuroblastoma carcinogenesis. Biochem Biophys Res Commun 394: 829–835.

Berry T, Luther W, Bhatnagar N, Jamin Y, Poon E, Sanda T *et al.* (2012). The ALK(F1174L) mutation potentiates the oncogenic activity of MYCN in neuroblastoma. Cancer Cell 22: 117–130.

Chen S, Bu G, Takei Y, Sakamoto K, Ikematsu S, Muramatsu T *et al.* (2007). Midkine and LDL-receptor-related protein 1 contribute to the anchorage-independent cell growth of cancer cells. J Cell Sci 120: 4009–4015.

Chen Y, Takita J, Choi YL, Kato M, Ohira M, Sanada M *et al.* (2008). Oncogenic mutations of ALK kinase in neuroblastoma. Nature 455: 971–974.

Dai LC, Wang X, Yao X, Min LS, Ping JL, He JF (2007a). Antisense oligonucleotides targeting midkine inhibit tumor growth in an in situ human hepatocellular carcinoma model. Acta Pharmacol Sin 28: 453–458.

Dai LC, Wang X, Yao X, Lu YL, Ping JL, He JF (2007b). Enhanced therapeutic effects of combined chemotherapeutic drugs and midkine antisense oligonucleotides for hepatocellular carcinoma. World J Gastroenterol 13: 1989–1994.

Dai LC, Yao X, Wang X, Niu SQ, Zhou LF, Fu FF *et al.* (2009). In vitro and in vivo suppression of hepatocellular carcinoma growth by midkine-antisense oligonucleotide-loaded nanoparticles. World J Gastroenterol 15: 1966–1972.

Englund C, Birve A, Falileeva L, Grabbe C, Palmer RH (2006). Miple1 and miple2 encode a family of MK/PTN homologues in Drosophila melanogaster. Dev Genes Evol 216: 10–18.

George RE, Sanda T, Hanna M, Fröhling S, Luther W 2nd, Zhang J *et al.* (2008). Activating mutations in ALK provide a therapeutic target in neuroblastoma. Nature 455: 975–978.

Güngör C, Zander H, Effenberger KE, Vashist YK, Kalinina T, Izbicki JR *et al.* (2011). Notch signaling activated by replication stress-induced expression of midkine drives epithelial-mesenchymal transition and chemoresistance in pancreatic cancer. Cancer Res 71: 5009–5019.

Hansford LM, Thomas WD, Keating JM, Burkhart CA, Peaston AE, Norris MD *et al.* (2004). Mechanisms of embryonal tumor initiation: distinct roles for MycN expression and MYCN amplification. Proc Natl Acad Sci USA 101: 12664–12669.

Heukamp LC, Thor T, Schramm A, De Preter K, Kumps C, De Wilde B *et al.* (2012). Targeted expression of mutated ALK induces neuroblastoma in transgenic mice. Sci Transl Med 4: 141ra91.

Hu R, Yan Y, Li Q, Lin Y, Jin W, Li H *et al.* (2010). Increased drug efflux along with midkine gene high expression in childhood B-lineage acute lymphoblastic leukemia cells. Int J Hematol 92: 105–110.

Huang P, Kishida S, Cao D, Murakami-Tonami Y, Mu P, Nakaguro M *et al.* (2011). The neuronal differentiation factor NeuroD1 downregulates the neuronal repellent factor Slit2 expression and promotes cell motility and tumor formation of neuroblastoma. Cancer Res 71: 2938–2948.

Huang Y, Hoque MO, Wu F, Trink B, Sidransky D, Ratovitski EA (2008). Midkine induces epithelial-mesenchymal transition through Notch2/Jak2-Stat3 signaling in human keratinocytes. Cell Cycle 7: 1613–1622.

Ibusuki M, Fujimori H, Yamamoto Y, Ota K, Ueda M, Shinriki S *et al.* (2009). Midkine in plasma as a novel breast cancer marker. Cancer Sci 100: 1735–1739.

Ikematsu S, Yano A, Aridome K, Kikuchi M, Kumai H, Nagano H *et al.* (2000). Serum midkine levels are increased in patients with various types of carcinomas. Br J Cancer 83: 701–706.

Ikematsu S, Nakagawara A, Nakamura Y, Sakuma S, Wakai K, Muramatsu T *et al.* (2003). Correlation of elevated level of blood midkine with poor prognostic factors of human neuroblastomas. Br J Cancer 88: 1522–1526.

Ikematsu S, Nakagawara A, Nakamura Y, Ohira M, Shinjo M, Kishida S *et al.* (2008). Plasma midkine level is a prognostic factor for human neuroblastoma. Cancer Sci 99: 2070–2074.

Ishiguro A, Akiyama T, Adachi H, Inoue J, Nakamura Y (2011). Therapeutic potential of anti-interleukin-17A aptamer: suppression of interleukin-17A signaling and attenuation of autoimmunity in two mouse models. Arthritis Rheum 63: 455–466.

Janoueix-Lerosey I, Lequin D, Brugières L, Ribeiro A, de Pontual L, Combaret V *et al*. (2008). Somatic and germline activating mutations of the ALK kinase receptor in neuroblastoma. Nature 455: 967–970.

Jia HL, Ye QH, Qin LX, Budhu A, Forgues M, Chen Y *et al*. (2007). Gene expression profiling reveals potential biomarkers of human hepatocellular carcinoma. Clin Cancer Res 13: 1133–1139.

Kadomatsu K, Muramatsu T (2004). Midkine and pleiotrophin in neural development and cancer. Cancer Lett 204: 127–143.

Kadomatsu K, Tomomura M, Muramatsu T (1988). cDNA cloning and sequencing of a new gene intensely expressed in early differentiation stages of embryonal carcinoma cells and in mid-gestation period of mouse embryogenesis. Biochem Biophys Res Commun 151: 1312–1318.

Kadomatsu K, Huang RP, Suganuma T, Murata F, Muramatsu T (1990). A retinoic acid responsive gene MK found in the teratocarcinoma system is expressed in spatially and temporally controlled manner during mouse embryogenesis. J Cell Biol 110: 607–616.

Kadomatsu K, Hagihara M, Akhter S, Fan QW, Muramatsu H, Muramatsu T (1997). Midkine induces the transformation of NIH3T3 cells. Br J Cancer 75: 354–359.

Kadomatsu K, Kishida S, Tsubota S (2013). The heparin-binding growth factor midkine: the biological activities and candidate receptors. J Biochem 153: 511–521.

Kang HC, Kim IJ, Park JH, Shin Y, Ku JL, Jung MS *et al*. (2004). Identification of genes with differential expression in acquired drug-resistant gastric cancer cells using high-density oligonucleotide microarrays. Clin Cancer Res 10: 272–284.

Kishida S, Mu P, Miyakawa S, Fujiwara M, Abe T, Sakamoto K *et al*. (2013). Midkine promotes neuroblastoma through Notch2 signaling. Cancer Res 73: 1318–1327.

Konishi N, Nakamura M, Nakaoka S, Hiasa Y, Cho M, Uemura H *et al*. (1999). Immunohistochemical analysis of midkine expression in human prostate carcinoma. Oncology 57: 253–257.

Lorente M, Torres S, Salazar M, Carracedo A, Hernández-Tiedra S, Rodríguez-Fornés F *et al*. (2011). Stimulation of the midkine/ALK axis renders glioma cells resistant to cannabinoid antitumoral action. Cell Death Differ 18: 959–973.

Maeda N, Ichihara-Tanaka K, Kimura T, Kadomatsu K, Muramatsu T, Noda M (1999). A receptor-like protein-tyrosine phosphatase PTPζ/RPTPβ binds a heparin-binding growth factor midkine. Involvement of arginine 78 of midkine in the high affinity binding to PTPζ. J Biol Chem 274: 12474–12479.

Mirkin BL, Clark S, Zheng X, Chu F, White BD, Greene M *et al*. (2005). Identification of midkine as a mediator for intercellular transfer of drug resistance. Oncogene 24: 4965–4974.

Mishima K, Asai A, Kadomatsu K, Ino Y, Nomura K, Narita Y *et al*. (1997). Increased expression of midkine during the progression of human astrocytomas. Neurosci Lett 233: 29–32.

Miyakawa S, Oguro A, Ohtsu T, Imataka H, Sonenberg N, Nakamura Y (2006). RNA aptamers to mammalian initiation factor 4G inhibit cap-dependent translation by blocking the formation of initiation factor complexes. RNA 12: 1825–1834.

Miyakawa S, Nomura Y, Sakamoto T, Yamaguchi Y, Kato K, Yamazaki S *et al*. (2008). Structural and molecular basis for hyperspecificity of RNA aptamer to human immunoglobulin G. RNA 14: 1154–1163.

Mossé YP, Laudenslager M, Longo L, Cole KA, Wood A, Attiyeh EF *et al*. (2008). Identification of ALK as a major familial neuroblastoma predisposition gene. Nature 455: 930–935.

Muramatsu H, Zou P, Suzuki H, Oda Y, Chen GY, Sakaguchi N *et al*. (2004). α4β1- and α6β1-integrins are functional receptors for midkine, a heparin-binding growth factor. J Cell Sci 117: 5405–5415.

Muramatsu T (2000). Protein-bound carbohydrates on cell-surface as targets of recognition: an odyssey in understanding them. Glycoconj J 17: 577–595.

Nakagawara A, Ohira M (2004). Comprehensive genomics linking between neural development and cancer: neuroblastoma as a model. Cancer Lett 204: 213–224.

Nakagawara A, Milbrandt J, Muramatsu T, Deuel TF, Zhao H, Cnaan A *et al*. (1995). Differential expression of pleiotrophin and midkine in advanced neuroblastomas. Cancer Res 55: 1792–1797.

Obata Y, Kikuchi S, Lin Y, Yagyu K, Muramatsu T, Kumai H (2005). Serum midkine concentrations and gastric cancer. Cancer Sci 96: 54–56.

Ota K, Fujimori H, Ueda M, Shiniriki S, Kudo M, Jono H *et al*. (2008). Midkine as a prognostic biomarker in oral squamous cell carcinoma. Br J Cancer 99: 655–662.

Rebbaa A, Chou PM, Mirkin BL (2001). Factors secreted by human neuroblastoma mediated doxorubicin resistance by activating STAT3 and inhibiting apoptosis. Mol Med 7: 393–400.

Reiff T, Huber L, Kramer M, Delattre O, Janoueix-Lerosey I, Rohrer H (2011). Midkine and Alk signaling in sympathetic neuron proliferation and neuroblastoma predisposition. Development 138: 4699–4708.

Sakamoto K, Bu G, Chen S, Takei Y, Hibi K, Kodera Y *et al*. (2011). Premature ligand-receptor interaction during biosynthesis limits the production of growth factor midkine and its receptor LDL receptor-related protein 1. J Biol Chem 286: 8405–8413.

Stoica GE, Kuo A, Powers C, Bowden ET, Sale EB, Riegel AT *et al*. (2002). Midkine binds to anaplastic lymphoma kinase (ALK) and acts as a growth factor for different cell types. J Biol Chem 277: 35990–35998.

Sueyoshi T, Jono H, Shinriki S, Ota K, Ota T, Tasaki M *et al*. (2012). Therapeutic approaches targeting midkine suppress tumor growth and lung metastasis in osteosarcoma. Cancer Lett 316: 23–30.

Takei Y, Kadomatsu K, Matsuo S, Itoh H, Nakazawa K, Kubota S *et al*. (2001). Antisense oligodeoxynucleotide targeted to Midkine, a heparin-binding growth factor, suppresses tumorigenicity of mouse rectal carcinoma cells. Cancer Res 61: 8486–8491.

Takei Y, Kadomatsu K, Itoh H, Sato W, Nakazawa K, Kubota S *et al*. (2002). 5′-,3′-inverted thymidine-modified antisense oligodeoxynucleotide targeting midkine. Its design and application for cancer therapy. J Biol Chem 277: 23800–23806.

Takei Y, Kadomatsu K, Yuasa K, Sato W, Muramatsu T (2005). Morpholino antisense oligomer targeting human midkine: its application for cancer therapy. Int J Cancer 114: 490–497.

Takei Y, Kadomatsu K, Goto T, Muramatsu T (2006). Combinational antitumor effect of siRNA against midkine and paclitaxel on growth of human prostate cancer xenografts. Cancer 107: 864–873.

Tomomura M, Kadomatsu K, Nakamoto M, Muramatsu H, Kondoh H, Imagawa K *et al*. (1990). A retinoic acid responsive gene, MK, produces a secreted protein with heparin binding activity. Biochem Biophys Res Commun 171: 603–609.

Tsutsui J, Kadomatsu K, Matsubara S, Nakagawara A, Hamanoue M, Takao S *et al*. (1993). A new family of heparin-binding growth/differentiation factors: increased midkine expression in Wilms' tumor and other human carcinomas. Cancer Res 53: 1281–1285.

Weiss WA, Aldape K, Mohapatra G, Feuerstein BG, Bishop JM (1997). Targeted expression of MYCN causes neuroblastoma in transgenic mice. EMBO J 16: 2985–2995.

Weiss WA, Godfrey T, Francisco C, Bishop JM (2000). Genome-wide screen for allelic imbalance in a mouse model for neuroblastoma. Cancer Res 60: 2483–2487.

Xu YY, Mao XY, Song YX, Zhao F, Wang ZN, Zhang WX *et al*. (2012). Midkine confers Adriamycin resistance in human gastric cancer cells. Tumour Biol 33: 1543–1548.

Ye C, Qi M, Fan QW, Ito K, Akiyama S, Kasai Y *et al*. (1999). Expression of midkine in the early stage of carcinogenesis in human colorectal cancer. Br J Cancer 79: 179–184.

Zage PE, Nolo R, Fang W, Stewart J, Garcia-Manero G, Zweidler-McKay PA (2011). Notch pathway activation induces neuroblastoma tumor cell growth arrest. Pediatr Blood Cancer 58: 682–689.