Table 1. Clinical data for the neuroblastoma cases in the present study

Case no.	Age	Stage	Diagnosis	Histology	*MYCN* amplification	Outcome
1	4 years 2 months	4	C	NBL poorly dif.	+	Alive
2	2 years 4 months	4	C	NBL poorly dif.	+	Alive
3	4 years	4	C	NBL poorly dif.	+	Alive
4	3 years	4	C	NBL poorly dif.	–	Alive
5	1 year 5 months	4	C	NBL poorly dif.	–	Alive
6	10 day	3	C	NBL dif.	–	Alive
7	4 years	4	C	NBL poorly dif.	–	Dead
8	4 years 2 months	4	C	NBL poorly dif.	–	Alive
9	2 years	3	C	GNB well dif.	–	Alive
10	10 years	4	C	NBL	–	Dead
11	4 years	4	C	NBL	+	Dead
12	3 years	3	C	NBL	+	Alive
13	11 years 9 months	4	C	NBL poorly dif.	–	Alive
14	6 months	3	MS	GNB	–	Alive
15	7 months	4	MS	NBL poorly dif.	–	Dead
16	4 years	4	C	NBL	+	Dead
17	4 years 9 months	4	C	NBL	–	Dead
18	7 months	4	MS	NBL	–	Alive
19	2 years	4	C	NBL poorly dif.	+	Alive
20	3 years	4	C	NBL	+	Dead
21	8 years	4	C	NBL poorly dif.	–	Alive
22	2 years 3 months	4	C	NBL	+	Alive
23	4 years	4	C	NBL	+	Dead
24	5 months	4	C	NBL	–	Alive
25	5 years	4	C	NBL	–	Dead
26	4 years 10 months	4	C	NBL	–	Alive
27	7 years	4	C	NBL poorly dif.	+	Dead
28	1 year 6 months	3	C	NBL	–	Alive
29	1 year 8 months	4	C	NBL	–	Alive
30	8 months	4	C	NBL	–	Alive

C, clinical; MS, mass screening program; NBL, neuroblastoma; NBL poorly dif., poorly differentiated neuroblastoma; GNB, ganglioneuroblastoma; GNB well dif., well-differentiated ganglioneuroblastoma.

Table 2. Neuroblastoma cell lines used in the present study

Cell line	MYCN amplification
CHP-134	–
GOTO	+
IMR-32	+
LAN-1	+
LAN-2	+
LAN-5	+
NB-1	–
NB-16	+
NB-19	+
NB-69	–
NH-12	+
SCMC-N2	+
SCMC-N4	+
SCMC-N5	+
SJNB-1	–
SJNB-2	+
SJNB-3	–
SJNB-4	+
SJNB-5	+
SJNB-6	+
SJNB-7	+
SJNB-8	+
SK-N-SH	–
TGW	+
UTP-N-1	+

Image 1.61 software (Wayne Rasband; National Institutes of Health, Bethesda, MD, USA).

Quantitative RT-PCR. To quantify the expression levels of *NEGR1*, real-time PCR (RQ-PCR) analysis was performed using the QuantiTect SYBR Green PCR kit (Qiagen, Tokyo, Japan) with an iCycler iQ real-time PCR detection system (Bio-Rad Japan, Tokyo, Japan). The primer sets used for the RQ-PCR are listed in Table S1 and the PCR conditions were as described previously.[13] For the purpose of normalization, relative expression levels were calculated by dividing the expression level of the respective gene by that of *β-actin*.

Mutational analysis of *MYEOV* and *NEGR1* genes. Genetic screening for *MYEOV* and *NERG1* genes in 25 cell lines was performed by denaturing HPLC (DHPLC) using the WAVE System Model 4500 (Transgenomic, Omaha, NE, USA), as described previously.[14] The primer sets used in the present study are listed in Table S1.

Bisulfate modification and methylation-specific PCR. Bisulfate modification of genomic DNA was performed as described previously.[15] For methylation-specific PCR (MSP), approximately 10 ng bisulfite-treated DNA was amplified with primers for both the methylated and unmethylated sequences. Reaction products were separated by electorophoresis on a 2.0% agarose gel. The primer sets for methylation-specific PCR analysis are listed in Table S1.

Knockdown of *MYEOV* using siRNA. The functional roles of the *MYEOV* gene in neuroblastoma cells was assessed using gene knockdown with siRNA.[16] The siRNA was designed and synthesized for silencing *MYEOV* (Invitrogen, Carlsbad, CA,

doi: 10.1111/j.1349-7006.2011.01995.x

USA). The siRNA duplex had the following sequences: 1132 sense, 5′-UCA ACG CCC ACU CUA AAG GCU UCU C-3′; and 1132 antisense, 5′-GAG AAG CCU UUA GAG UGG GCG UUG A-3′. A chemically synthesized non-silencing siRNA duplex that had no known homology to any mammalian gene was used as a control for non-specific silencing events and had the following sequences: sense, 5′-UUC UCC GAA CGU GUC ACG UdT dT-3′; and antisense, 5′-ACG UGA CAC GUU CGG AGA AdT dT-3′. Gene knockdown was achieved in NB-19, CHP-134 and PF-SK-1 cells using HiPerFect transfection reagent (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions.

Transient transfection. The expression vector (pME18S) containing the full-length *EcoRI-Xba1* fragment of the *NEGR1* cDNA was transfected into NB-19, SJNB-7, and PF-SK-1 cells using the lipofection method according to the manufacturer's instructions (Qiagen).[11] Briefly, 1.5×10^5 cells were seeded in a six-well plate and incubated in 1.6 mL RPMI 1640 (Gibco-BRL) with 10 μL Effectance reagent (Qiagen), 3.2 μL Enhancer (1:8) (Qiagen), 10 μL Effectene (Qiagen), and 0.4 μg expression vector. Cells were counted 72 h after transfection.

Statistical analysis. Expression of the *NEGR1* gene was compared between favorable and unfavorable cases of neuroblastomas using the Mann–Whitney *U*-test. Exact 95% confidence intervals (CI) of the proportions were calculated on the basis of binomial distribution. The Kruskal–Wallis test was used to compare the functional effects of *MYEOV* inhibition and *NEGR1* expression in neuroblastoma cells.

Results

Gain and high-grade amplification of 11q13 involving the *MYEOV* locus in neuroblastoma. In the present series, gains of chromosome 11q13 were detected in multiple neuroblastoma cases.[3] Within this gain, a high-grade amplification was found in a single case with Stage 4 disease (Case 22; Fig. 1a). The critical amplicon that had minimum overlapping amplification/gain was found in a 340-kb region exclusively containing *MYEOV*, located 360 kb upstream from the *CCND1* locus[7] (Fig. 1a). Previously, *MYEOV* had been identified as a putative transforming gene based on the NIH/3T3 tumorigenicity assay[9] and was shown to be highly expressed in a subset of multiple myelomas harboring t(11;14)(q13;q32).[7] We further examined the expression patterns of *MYEOV* in a total of 45 neuroblastoma samples using semi-quantitative RT-PCR analysis, in which 11 of 25 cell lines (44%) and seven of 20 fresh tumors (35%) showed higher expression levels of *MYEOV* compared with the median expression level (*MYEOV/β-actin* signal intensity = 1.4; Fig. 1b). Although most tumors exhibited increased expression of both *CCND1* and *MYEOV*, Case 22 showed high expression of *MYEOV* but not *CCND1* (Fig. 1c). Mutational analysis of the coding region of *MYEOV* was also performed in 25 cell lines, but no tumor-specific mutations were detected.

Homozygous deletion on 1p31 detected in neuroblastoma. Detection of homozygous deletions was also of interest because they provide an important clue in pinpointing tumor suppressor loci. In an allele-specific copy number analyzer for GeneChip (CNAG) and allele-specific copy number analysis using anonymous references (AsCNAR), homozygous deletions could be identified as the loss of both parental alleles, even in the presence of significant components of normal tissues.[3] In the present study, 70 homozygous deletions were identified at 50 independent loci in the neuroblastoma samples. Unfortunately, we were not able to completely exclude the possibility that some may represent copy number variations (CNV) rather than real homozygous deletions, because paired DNA was available only in four primary neuroblastoma cases and many homozygous deletions were found in established neuroblastoma cell

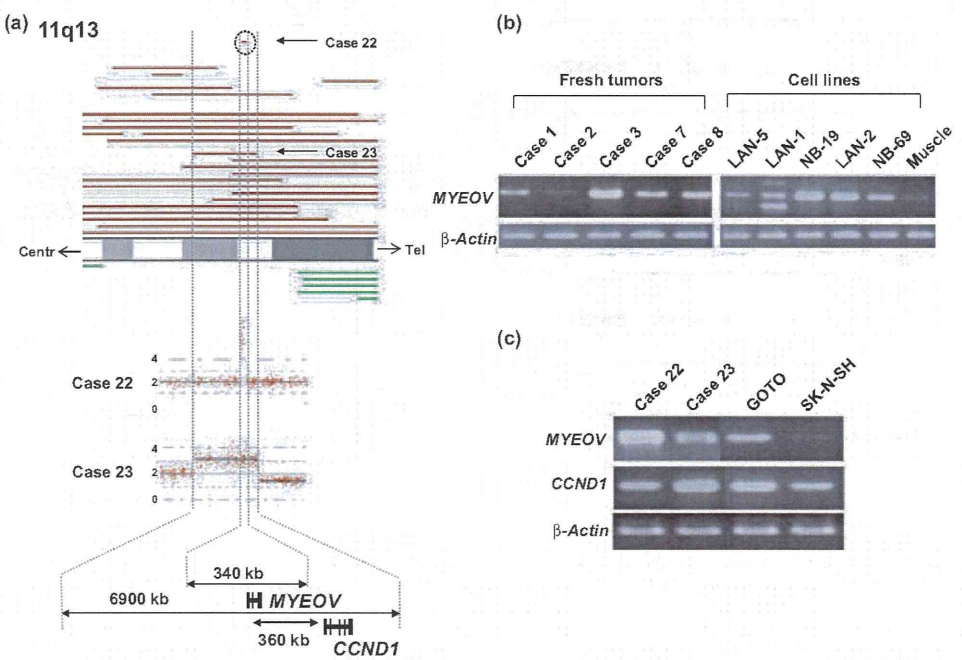

Fig. 1. Gains and high-grade amplification on chromosome 11q13 in neuroblastoma. (a) A common 340-kb region having copy number (CN) gains contains a single known gene, *MYEOV*. In addition, *CCND1* is frequently contained in CN gains at 11q13, but mapped outside the minimum region of common CN gains. Red bars, gains (3 < CN < 5); green bars, losses (CN = 1); light red bar (circled), high-grade amplification (CN ≥ 5). (b) Representative results of *MYEOV* expression in fresh tumors and cell lines (RNA from normal muscle was used as a control). (c) Expression of *MYEOV* and *CCND1* in Cases 22 and 23 (RNA from normal muscle was used as a control). The expression of *MYEOV* in Case 22 tended to be higher than that in Case 23. tel, telomere; centr, centromere.

lines. Complete loss of genetic material at eight loci was confirmed by genomic PCR (data not shown).

Of the 70 homozygous deletions identified, we focused on a homozygous deletion involving a 370-kb region at 1p31 in NB-19. This region contains a part of *NEGR* (exon 1 and a part of intron 1), a unique candidate target gene, which was also disrupted by a translocation in another cell line, namely SJNB-6 (Fig. 2a,b). Because *NEGR1* encodes a member of the IgLON family of cell adhesion molecule sand has been reported to be a putative tumor suppressor gene in ovarian cancer,[8] we examined its expression in neuroblastoma cases in the present study to evaluate the clinical impact of *NEGR1* expression. As shown in Figure 2(c), *NEGR1* expression was absent or very low in 10 of 25 (40%) cell lines, as determined by semi-quantitative RT-PCR (Fig. 2c). In quantitative RT-PCR analysis using fresh tumor samples (20 fresh advanced-stage tumors and an additional 20 cases of early stage tumors), the expression of the *NEGR1* gene was significantly lower in advanced-stage tumors compared with early stage tumors ($P = 0.0041$; Fig. 2c). Similarly, the expression of the *NEGR1* gene was significantly lower in patients who died compared with patients who survived ($P = 0.018$; Fig. S1). Mutation analysis was also performed in neuroblastoma cell lines, but no tumor-specific mutations were detected. Methylation analysis of the promoter region of *NEGR1* using 10 neuroblastoma cell lines without *NEGR1* expression did not reveal any tumor-specific methylation pattern in neuroblastoma cell lines or fresh neuroblastoma samples (data not shown).

Functional analyses of *MYEOV* and *NEGR1* in neuroblastoma cell lines. We further evaluated the oncogenic potential of *MYEOV* using siRNA-mediated gene knockdown in the NB-19 cell line, which highly expresses *MYEOV*. As shown in Figure 3(a,b). When *MYEOV* expression was suppressed by siRNA, NB-19 cells exhibited retarded growth compared with the growth of control cells ($P = 0.0027$), indicating that MYEOV positively regulates cell proliferation (Fig. 3a,b). Similar results were obtained in CHP-134 and PF-SK-1 cells (Fig. S2). To assess the tumor suppressor function of *NEGR1* in neuroblastoma cells, we generated an *NEGR1* expression vector that was transiently transfected into NB-19 cells, in which *NEGR1* is homozygously deleted. Expression of *NEGR1* significantly suppressed the proliferation of NB-19 cells compared with mock transfection ($P = 0.019$; Fig. 3c,d). In addition, the *NEGR1* expression vector was transiently transfected into SJNB-7 and PF-SK-1 cells, in which *NEGR1* expression is absent. Following transfection into these cell lines, profound inhibition of cell proliferation was observed for both SJNB-7 and PF-SK-1 cells expressing *NEGR1* (Fig. S3).

Discussion

In the present study, we showed that *MYEOV* and *NEGR1* are candidate gene targets of 11q13 gain and 1p31 deletion, respectively, in a neuroblastoma subset. To our knowledge, this is the first report to describe aberrations of *MYEOV* and *NEGR1* in neuroblastoma.

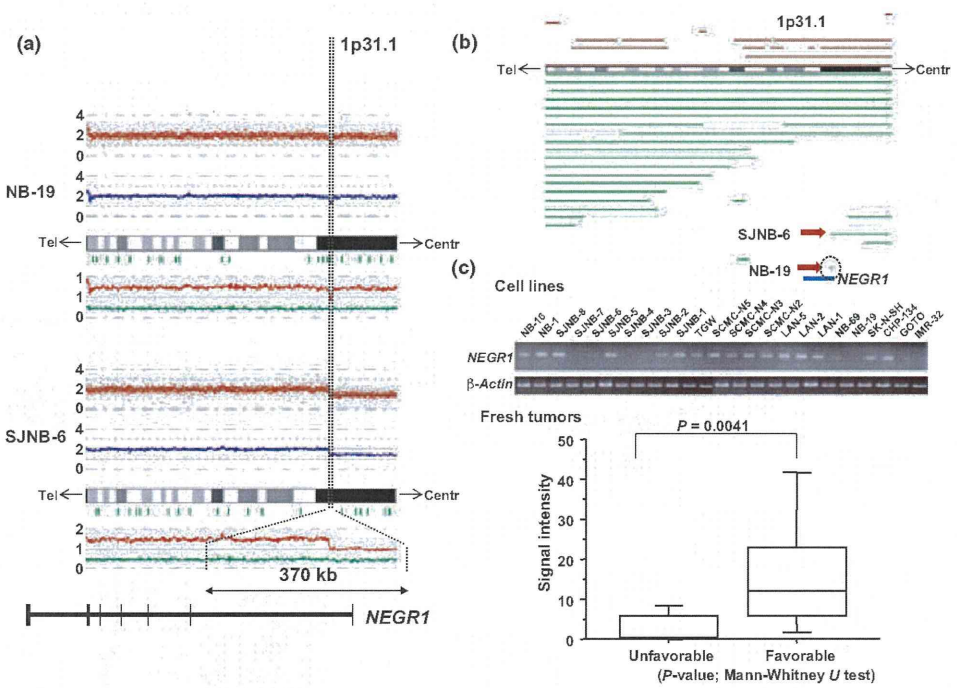

Fig. 2. *NEGR1* as a candidate tumor suppressor gene in neuroblastoma. (a) Deletion mapping of 1p31.1 disclosed a homozygous deletion spanning a 370-kb region in the NB-19 cell line, which contains part of *NEGR1* as the only structural gene. The *NEGR1* gene is also disrupted in intron 1 by the breakpoint of a segmental duplication at 1p31.1 in another neuroblastoma cell line (SJNB-6). For each panel, total copy numbers (tCN; red dots), moving averages of tCN for five consecutive single nucleotide polymorphisms (SNP; blue line), an ideogram of the relevant chromosome, the location of heterozygous SNP calls (green bars), and allele-specific copy numbers (AsCN) averaged for five consecutive SNP (red and green lines for larger and smaller alleles, respectively) are plotted. Note that the CN are expressed in terms of "observed" signal ratios between tumor and reference samples, where the baseline is adjusted to 2 for tCN plots and to 1 for AsCN. (b) Summary of CN abnormalities of 1p31.1 in neuroblastoma. Red bars, gains ($3 < CN < 5$); green bars, losses ($CN = 1$); light green bar (circled), homozygous deletion ($CN = 0$). A homozygous deletion detected in NB-19 and a chromosomal rearrangement detected in SJNB-6 are indicated by the red arrows. The location of *NEGR1* is shown by the blue line. (c) *NEGR1* expression in neuroblastoma. Top panel: representative result of *NEGR1* expression in neuroblastoma cell lines showing frequently reduced expression levels in a subset of neuroblastoma cell lines. Bottom graph: expression of the *NEGR1* gene as measured by quantitative PCR was significantly lower in tumors with an unfavorable outcome than in tumors with a favorable outcome ($P = 0.0041$, Mann–Whitney *U*-test). tel, telomere; centr, centromere.

doi: 10.1111/j.1349-7006.2011.01995.x

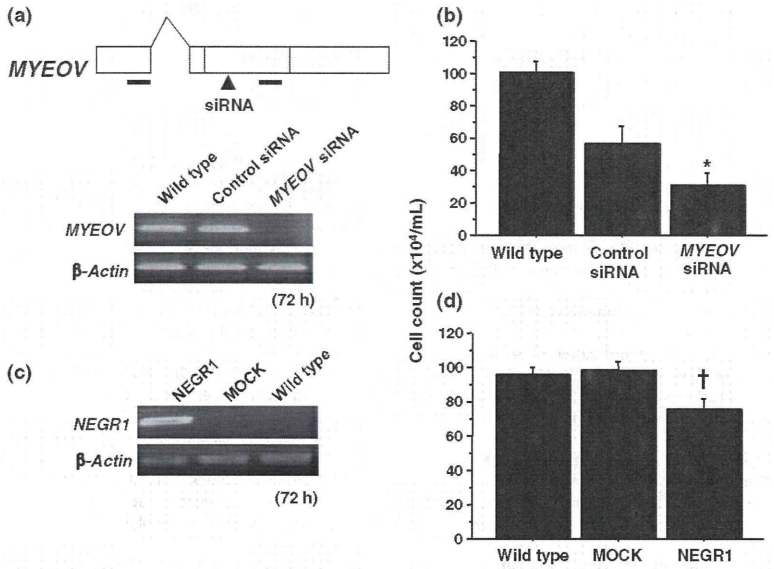

Fig. 3. Effect of *MYEOV* inhibition by siRNA on cell growth and effect of *NEGR1* on cell growth in neuroblastoma cells. (a) Confirmation of siRNA-mediated gene knockdown using semi-quantitative RT-PCR analysis. Following siRNA treatment, *MYEOV* mRNA was absent in treated cells; however, abundant *MYEOV* expression was detected in wild-type and control cells. (b) Effect of *MYEOV* inhibition by siRNA transfected into NB-19 cells on cell growth. Cell growth was impaired cell growth in siRNA-transfected cells compared with that of control cells (*P = 0.0027, Kruskal–Wallis test). (c) Analysis (RT-PCR) of NB-19 cells transfected with the pME18S vector. Mock-transfected and wild-type cells were used as controls. (d) The growth of cells transiently expressing *NEGR1* was impaired compared with that of mock-transfected and wild-type cells (†P = 0.019, Kruskal–Wallis test).

Initially, *MYEOV* was reported as a gene that was possibly co-overexpressed with *CCND1* in some cases of multiple myeloma with t(11;14)(q13;q32); later, it was shown to be co-amplified and co-overexpressed with *CCND1* in a subset of esophageal squamous cell carcinomas, breast cancers, gastric cancers, and colorectal cancers.[7,17–19] Although the major genetic targets of these rearrangements and amplifications have been shown to be *CCND1*, in some breast cancer cases the 11q13 amplicon exclusively contained *MYEOV* and not *CCDN1*, suggesting a *CCDN1*-independent oncogenic role for *MYEOV*.[7] The oncogenic role of *MYEOV* has also been investigated in functional studies, showing that *in vitro* siRNA-mediated knockdown of *MYEOV* resulted in inhibition of proliferation, invasion, and migration of colorectal cancer cell lines.[19] In our neuroblastoma cases, *MYEOV* was overexpressed in approximately 30% of primary neuroblastoma cases, with seven cases showing gain/amplification of MYEOV. We also confirmed that *MYEOV* was the only gene found in the common gain/amplicon at 11q13 and that proliferation of neuroblastoma cell lines was inhibited by siRNA-mediated *MYEOV* knockdown, supporting an oncogenic role for *MYEOV* in some neuroblastoma cases. Although several studies have revealed that *MYEOV* amplification is associated with poor prognosis in multiple myeloma, esophageal squamous cell carcinoma, and breast cancer,[7,18,20] the clinical impact of *MYEOV* gain/amplification or overexpression in neuroblastoma is unclear and requires further evaluation.

The *NEGR1* gene is a single gene found in one of the recurrent deletions at 1p31. Although the *NEGR1* locus is known as one of the most common CNV regions,[21] we also identified a neuroblastoma cell line in which *NEGR1* was disrupted in by gene rearrangement, supporting the fact that *NEGR1* is one of the target genes in neuroblastoma. In ovarian cancer, *NEGR1* is a putative tumor suppressor gene encoding one of the IgLON cell adhesion family members, namely OPCML, and it plays a central role in the establishment and remodeling of the central nervous system.[22] Notably, OPCML has been shown to exhibit functional characteristics of a tumor suppressor gene in epithelial ovarian cancer.[23] In our analysis, expression of *NEGR1* was substantially reduced in 43% of advanced-stage tumors without 1p31 deletions/rearrangement. In addition, re-expression of *NEGR1* in the NB-19 cell line with homozygous deletion of *NEGR1*, as well as in other neuroblastoma cell lines that did not express *NEGR1*, resulted in the inhibition of cell growth, suggesting that *NEGR1* is a candidate tumor suppressor gene in neuroblastoma and may have possible prognostic value. Although expression of OPCML in ovarian cancers is suppressed or reduced mainly through epigenetic mechanisms,[23] tumor-specific methylation was not detected in neuroblastoma cells in the present study. The mechanisms for the absence of *NEGR1* in the tumors without homozygous deletion, mutation, and methylation were not clear in the present study. We cannot role out the possibility that mutations are harbored in the promoter region of *NEGR1* with consequent gene inactivation. Furthermore, *NEGR1* was often heterozygously deleted, but not mutated or methylated, in neuroblastoma; most deletions occur in tumors at advanced stages, suggesting that *NEGR1* has haploinsufficient effects on advanced disease in neuroblastoma.

In conclusion, the results of the present study suggest that *MYEOV* at 11q13 and *NEGR1* at 1p31 are functional gene targets in a subset of neuroblastoma. Further studies on both genes will expand these pathways and provide insights into the progression of neuroblastoma, as well as possibly enabling the development of novel therapeutics based on targeting MYEOV and NEGR1 in neuroblastoma.

Acknowledgments

The authors thank Mrs Matsumura M, Mrs Hoshino N, Mrs Yin Y and Mrs Saito F for their excellent technical assistance. The authors also express their appreciation to Drs A.T. Look (Harvard Medical University, Boston, MA, USA) and Dr. A. Inoue (St. Jude Children's Research Hospital, Memphis, TN, USA), for their generous gifts of neuroblastoma cell lines. This work was supported by Research on Measures for Intractable Diseases, Health, and Labor Sciences Research Grants; the

Ministry of Health, Labor and Welfare via a grant for Research on Health Sciences focusing on Drug Innovation; by the Japan Health Sciences Foundation; and by the Core Research for Evolutional Science and Technology, Japan Science and Technology Agency.

Disclosure Statement

The authors have no conflicts of interest.

References

1 Brodeur GM. Neuroblastoma: biological insights into a clinical enigma. *Nat Rev Cancer* 2003; **3**: 203–16.
2 Brodeur GM, Seeger RC, Schwab M, Varmus HE, Bishop JM. Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage. *Science* 1984; **224**: 1121–4.
3 Chen Y, Takita J, Choi YL *et al.* Oncogenic mutations of ALK kinase in neuroblastoma. *Nature* 2008; **455**: 971–4.
4 Mosse YP, Laudenslager M, Longo L *et al.* Identification of ALK as a major familial neuroblastoma predisposition gene. *Nature* 2008; **455**: 930–5.
5 Janoueix-Lerosey I, Lequin D, Brugieres L *et al.* Somatic and germline activating mutations of the ALK kinase receptor in neuroblastoma. *Nature* 2008; **455**: 967–70.
6 George RE, Sanda T, Hanna M *et al.* Activating mutations in ALK provide a therapeutic target in neuroblastoma. *Nature* 2008; **455**: 975–8.
7 Janssen JW, Cuny M, Orsetti B *et al.* MYEOV: a candidate gene for DNA amplification events occurring centromeric to CCND1 in breast cancer. *Int J Cancer* 2001; **102**: 608–14.
8 Ntougkos E, Rush R, Scott D *et al.* The IgLON family in epithelial ovarian cancer: expression profiles and clinicopathologic correlates. *Clin Cancer Res* 2005; **11**: 5764–8.
9 Brecht M, Steenvoorden AC, Luf S, Bartram CR, Janssen JW. Rearrangement and expression of myeov and hst in NIH/3T3 transfectants: a caveat for the interpretation of DNA transfection analyses. *Oncol Rep* 2007; **17**: 1127–31.
10 Smith EI, Haase GM, Seeger RC, Brodeur GM. A surgical perspective on the current staging in neuroblastoma: the International Neuroblastoma Staging System proposal. *J Pediatr Surg* 1989; **24**: 386–90.
11 Takita J, Hayashi Y, Nakajima T *et al.* The p16 (CDKN2A) gene is involved in the growth of neuroblastoma cells and its expression is associated with prognosis of neuroblastoma patients. *Oncogene* 1998; **17**: 3137–43.
12 Inoue HK, Shirao T. Neurite formation induced in neuroblastoma cells and genetically altered non-neuronal cells. *J Electron Microsc* 1997; **46**: 497–502.
13 Takita J, Ishii M, Tsutsumi S *et al.* Gene expression profiling and identification of novel prognostic marker genes in neuroblastoma. *Genes Chromosom Cancer* 2004; **40**: 120–32.
14 Donohoe TJ, Sintim HO, Sisangia L *et al.* Utility of the ammonia-free Birch reduction of electron-deficient pyrroles: total synthesis of the 20s proteasome inhibitor, clasto-lactacystin beta-lactone. *Chemistry* 2005; **11**: 4227–38.
15 Takita J, Yang HW, Chen YY *et al.* Allelic imbalance on chromosome 2q and alterations of the caspase 8 gene in neuroblastoma. *Oncogene* 2001; **20**: 4424–32.
16 Moss AC, Lawlor G, Murray D *et al.* ETV4 and Myeov knockdown impairs colon cancer cell line proliferation and invasion. *Biochem Biophys Res Commun* 2006; **345**: 216–21.
17 Janssen JW, Vaandrager JW, Heuser T *et al.* Concurrent activation of a novel putative transforming gene, myeov, and cyclin D1 in a subset of multiple myeloma cell lines with t(11;14)(q13;q32). *Blood* 2000; **95**: 2691–8.
18 Carneiro A, Isinger A, Karlsson A *et al.* Prognostic impact of array-based genomic profiles in esophageal squamous cell cancer. *BMC Cancer* 2008; **8**: 98.
19 Lawlor G, Doran PP, MacMathuna P, Murray DW. MYEOV (myeloma overexpressed gene) drives colon cancer cell migration and is regulated by PGE_2. *J Exp Clin Cancer Res* 2010; **29**: 81.
20 Moreaux J, Hose D, Bonnefond A *et al.* MYEOV is a prognostic factor in multiple myeloma. *Exp Hematol* 2010; **38**: 1189–98.
21 Jarick I, Vogel CI, Scherag S *et al.* Novel common copy number variation for early onset extreme obesity on chromosome 11q11 identified by a genome-wide analysis. *Hum Mol Genet* 2011; **20**: 840–52.
22 Funatsu N, Miyata S, Kumanogoh H *et al.* Characterization of a novel rat brain glycosylphosphatidylinositol-anchored protein (Kilon), a member of the IgLON cell adhesion molecule family. *J Biol Chem* 1999; **274**: 8224–30.
23 Sellar GC, Watt KP, Rabiasz GJ *et al.* OPCML at 11q25 is epigenetically inactivated and has tumor-suppressor function in epithelial ovarian cancer. *Nat Genet* 2003; **34**: 337–43.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. *NEGR1* expression in 30 neuroblastoma cases.

Fig. S2. Effect of siRNA inhibition of *MYEOV* on cell growth in CHP-134 and PF-SK-1 cells.

Fig. S3. Effect of *NEGR1* on cell growth in the neuroblastoma cell lines PF-SK-1 and SJNB-7.

Table S1. Primer sequences used in the present study.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

doi: 10.1111/j.1349-7006.2011.01995.x

Cancer Science
The official journal of the Japanese Cancer Association

Review Article

Oncogenic mutations of *ALK* in neuroblastoma

Seishi Ogawa,[1,2,6] Junko Takita,[3,4] Masashi Sanada[1] and Yasuhide Hayashi[5]

[1]Cancer Genomics Project, The University of Tokyo, Tokyo; [2]Core Research for Evolutional Science and Technology, Exploratory Research for Advanced Technology, Japan Science and Technology Agency, Saitama; [3]Department of Pediatrics, The University of Tokyo, Tokyo; [4]Cell Therapy and Transplantation Medicine, The University of Tokyo, Tokyo; [5]Gunma Children's Medical Center, Gunma, Japan

(Received October 21, 2010/Revised November 26, 2010/Accepted November 28, 2010/Accepted manuscript online December 9, 2010)

Neuroblastoma is one of the most common solid cancers among children. Prognosis of advanced neuroblastoma is still poor despite the recent advances in chemo/radiotherapies. In view of improving the clinical outcome of advanced neuroblastoma, it is important to identify the key molecules responsible for the pathogenesis of neuroblastoma and to develop effective drugs that target these molecules. Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase, initially identified through the analysis of a specific translocation associated with a rare subtype of non-Hodgkin's lymphoma. Recently it was demonstrated that *ALK* is frequently mutated in sporadic cases with advanced neuroblastoma. Moreover, germline mutations of *ALK* were shown to be responsible for the majority of hereditary neuroblastoma. ALK mutants found in neuroblastoma show constitutive active kinase activity and oncogenic potentials. Inhibition of ALK in neuroblastoma cell lines carrying amplified or mutated *ALK* alleles results in compromised downstream signaling and cell growth, indicating potential roles of small molecule ALK inhibitors in the therapeutics of neuroblastoma carrying mutated ALK kinases. (*Cancer Sci* 2011; 102: 302–308)

Neuroblastoma is a malignant embryonal neoplasm arising from developing neural crest tissues.[1] It commonly affects younger children, where the median age of diagnosis is 17 months and approximately 90% of the patients are <4 years old. In the United States, the incidence of neuroblastoma is estimated to be one in 7000 births, although the incidence calculated from the mass screening program in Japan was as high as 29.80 cases per 100 000 births, which is significantly higher than the estimation in the prescreening cohort (11.56 cases per 100 000 births).[2] It is the third most common cancer in childhood after leukemia and brain tumors, accounting for 7–11% of all pediatric cancers.[3] The presentation and following clinical courses of neuroblastoma are highly variable, ranging from a solitary localized mass with no apparent clinical symptoms to widely disseminated diseases presenting with severe systemic illness.[1] While some tumors undergo spontaneous regression without therapy, approximately 60–70% of high-risk neuroblastoma patients are resistant to any therapies currently available and succumb to death,[4–6] even though a substantial improvement in 5-year survival rates has been obtained for a subset of advanced tumors through the development of multimodal chemo/radiotherapies during the past several decades.[1] Thus, one of the urgent problems in the current neuroblastoma treatment would be to develop rational and effective therapeutic strategies for the high-risk neuroblastoma cases based on their molecular pathogenesis.

On the other hand, during the past three decades, little advancement has been made in the understanding of neuroblastoma pathogenesis in terms of critical gene targets, except for the identification of frequent *MYCN* amplification.[7] Amplification of the *MYCN* gene is found in approximately 20% of neuroblastoma, especially in advanced diseases, and has been consistently associated with poor prognosis.[8,9] Although *MYCN* amplification is a critical genetic event in neuroblastoma development,[10] it encodes a transcription factor and thus may not be a plausible pharmacological target for therapeutics. Recently, several groups independently discovered activating mutations of the *ALK* gene in the majority of familial neuroblastoma and also in a subset of sporadic neuroblastoma cases.[11–14] Given that the mutated ALK kinases are well-tractable targets for small-molecule kinase inhibitors, the discovery draws attention in the field of neuroblastoma research. In this review, we provide a brief overview of the role of *ALK* mutations in neuroblastoma pathogenesis and their implication in future therapeutics.

Genetic analysis of familial neuroblastoma

One of the first clues to identifying the novel genetic target of neuroblastoma was obtained from a linkage study of neuroblastoma-prone families. It was recognized that approximately 1–2% of newly diagnosed neuroblastoma cases occur within families (familial/hereditary neuroblastoma), indicating the existence of dominantly acting neuroblastoma susceptibility gene(s),[15–19] although previous linkage studies, in an attempt to identify the susceptibility locus, failed to provide a reproducible result due to the insufficient power of the studies.[20–22] Germline mutations of the paired-like homeobox 2B (*PHOX2B*) gene at 4p12 was reported to be responsible for neuroblastoma predisposition, but they were mostly related to a rare form of familial neuroblastoma associated with congenital central hypoventilation syndrome (CCHS) and/or Hirschsprung disease, with rare somatic mutations.[23–26] Recently, researchers at the Pennsylvania University analyzed 20 neuroblastoma pedigrees for linkage using approximately 6000 genetic markers, and mapped a candidate neuroblastoma susceptibility locus to the 2p region between rs18621106 and rs2008535, which contains 104 genes including *MYCN* and *ALK*.[11] Through a resequencing analysis of the *ALK* exons within the pedigrees they identified germline mutations of the *ALK* gene in >90% of the pedigrees that co-segregated with neuroblastoma development within the families, clearly demonstrating that the germline *ALK* mutations are responsible for the susceptibility to the development of hereditary neuroblastoma in the majority of the cases.[11,12] Moreover, the subsequent analysis of *ALK* mutations in sporadic neuroblastoma cases identified a subset of sporadic neuroblastoma cases carrying acquired/germline mutations of *ALK*, which was also reported independently by other groups.[12–14,27]

[6]To whom correspondence should be addressed.
E-mail: sogawa-tky@umin.ac.jp

doi: 10.1111/j.1349-7006.2010.01825.x

Genome-wide copy number scanning of neuroblastoma

These groups conducted genome-wide copy number analyses of neuroblastoma using comparative genomic hybridization (CGH) arrays[12] or single nucleotide polymorphism (SNP) arrays.[11,14,27,28] With thousands to half-a-million genetic probes, both platforms enabled high-throughput detection of subtle genetic changes occurring in tumor genomes.[29,30] Neuroblastoma genomes show characteristic copy number changes that involve large chromosomal segments, including gains of 17q, 1q, 2p and 11p, and losses of 1p, 3p and 11q, which, like other human cancers, collectively comprise a unique genomic profile of neuroblastoma.[11,12,14] High-level amplifications, which usually involve discrete chromosomal regions <1 Mb in length, occurred in approximately 30% of neuroblastoma cases. Approximately 90% of the high-level amplifications in neuroblastoma were centered on the *MYCN* locus at 2p24, whereas other amplicons rarely mutually overlapped, except for the amplifications at 2p23, which exclusively involved the *ALK* locus in common[12,14,28] (Fig. 1).

High-level amplification of the *ALK* gene and aberrantly activated ALK signaling in neuroblastoma was first described by Osajima-Hakomori *et al.*[31] in two neuroblastoma-derived cell lines and a single case of primary neuroblastoma. The genome-wide copy number studies confirmed their finding, in which the frequency of *ALK* amplifications is reported to occur in 3–5% of primary neuroblastoma cases.[11,12,14] Subsequent resequencing studies of *ALK* coding exons disclosed non-synonymous nucleotide substitutions of *ALK* in a subset of sporadic neuroblastoma cases and also of neuroblastoma-derived cell lines with mutation rates of approximately 6–11% and approximately 30%, respectively. Amplified *ALK* alleles, as a rule, did not harbor additional mutations, although in rare cases mutated ALK alleles were amplified.

Genetic abnormalities of the *ALK* gene in human cancers

ALK was initially isolated as a partner of the fusion gene generated by t(2;5)(q23;q35) translocation, which is characteristic of anaplastic large cell lymphoma (ALCL), a rare subtype of non-Hodgkin's lymphoma.[32,33] *ALK* encodes an orphan receptor tyrosine kinase with an apparent molecular mass of 220 kDa. Jelly belly,[34] and pleiotrophin[35] and midkine[36] have been postulated as putative ALK ligands in *Drosophila* and mammalians, respectively, but a dispute about the authentic ligands of ALK still remains. ALK has an extracellular domain that is highly similar to LTK and, together with IGF-1R and c-Ros kinases, belongs to the insulin family of proteins.[37] Expression of ALK is largely restricted to neural tissues and is most abundant in the neonatal brain and, to a lesser extent, in the adult brain.[38–41] In the developing brain, the highest expression was found in the thalamus, mid-brain, olfactory bulb and selected parts of cranial and dorsal ganglia.[38,39] It is of particular note that high frequencies of ALK expression were reported in primary neuroblastoma specimens (22 out of 24 samples) and in other tumor cell lines derived from neuroectdermal tumors including neuroblastoma (13 out of 29 cell lines).[42] These expression patterns of ALK suggest its primary role in normal neural development as well as the pathogenesis of neuroblastoma, although *ALK*-deficient mice seem to show apparently normal develop.[37]

In t(2;5)(q23;q35) translocation, the carbonyl terminal of ALK that contains a kinase domain is fused with nucleophosmin (NPM), generating NPM/ALK fusion protein. *ALK* was also shown to participate in the generation of different fusion genes with a variety of partner genes in ALCL,[43–47] inflammatory fibroblastic tumor,[43,48–52] squamous cell carcinoma of the esophagus[53] and non-small-cell lung cancers (NSCLC).[54,55] In NSCLC, *ALK* was reported to be fused with EML4 to generate EML4–ALK fusion protein as a result of inv(2)(p21p23), which is found in 6% of the NSCLC cases[55] (Fig. 2).

These ALK-containing fusion proteins invariably show constitutive kinase activity and transform NIH3T3 cells and/or confer growth factor independence to 32D and/or Ba/F3 cells.[56–58] When bone marrow cells were retrovirally transduced with *NPM–ALK* and transplanted into mice, they developed B-cell lymphoma within 4 months.[58] The critical role of ALK fusion proteins in neoplastic evolution has been further demonstrated

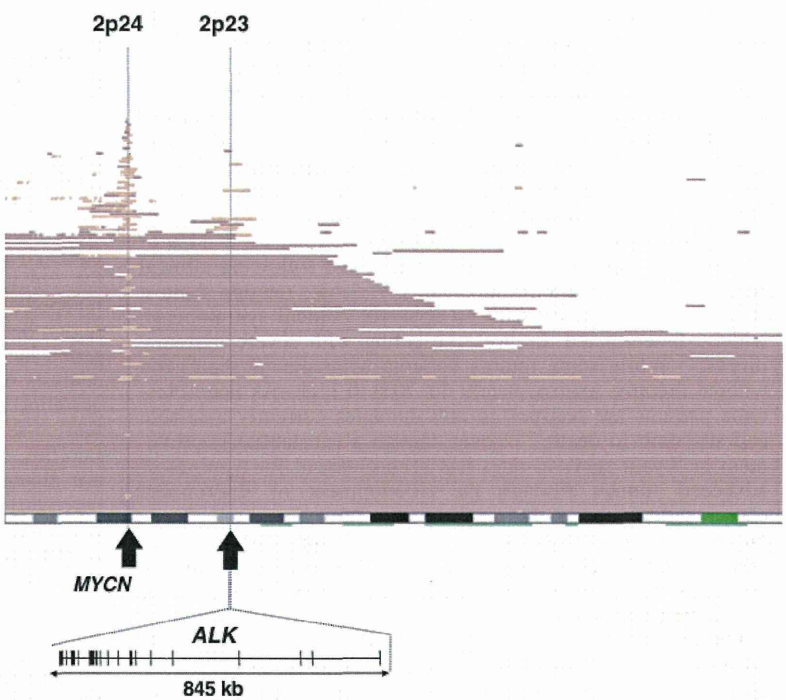

Fig. 1. Copy number gains and high-level amplifications in the short arm of chromosome 2 in neuroblastoma. Each horizontal line indicates a region showing a simple copy number (CN) gain (CN < 5; thick red) and high-level amplification (CN > 5; thin red) in each case. The majority of high-level amplifications involved the *MYCN* locus at 2p24, while the other group of amplicons is found at 2p23, which exclusively contains the *ALK* locus.

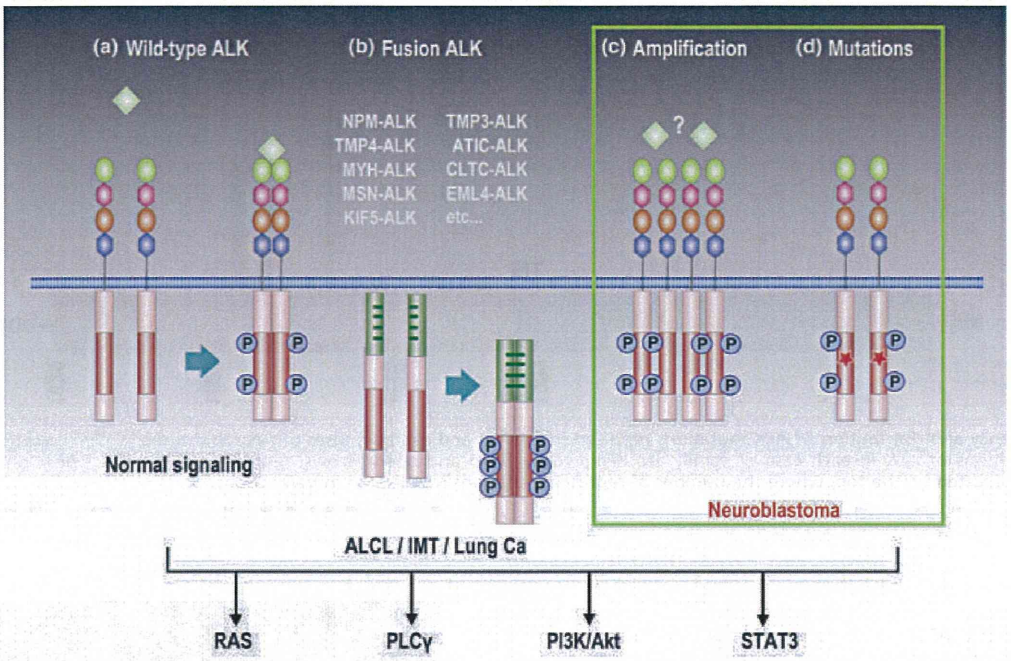

Fig. 2. Aberrant activation of ALK in human cancers. (a) Ligand-dependent physiological activation of wild-type ALK. (b) Fusion ALK kinases found in anaplastic large cell lymphoma (ALCL) and non-small-cell lung cancer (Lung Ca), such as NPM–ALK and EML4–ALK, self-dimerize through their N-terminal domains derived from fusion partners, leading to their transphosphorylation and constitutive activation of the kinase. In a subset of neuroblastoma, aberrant activation of ALK occurs by gene amplification (c) or somatic/germline mutations (d). Activated ALK transmits constitutive signals through downstream pathways, which is thought to be important for tumorigenesis. IMT indicates inflammatory myofibroblastic tumor.

using transgenic mouse models with *ALK* fusion genes; mice carrying *NPM–ALK* or *EML4–ALK* transgenes under *Vav* or *CD4*, or *surfactant protein C* promoter develop aggressive lymphoma or adenocarcinoma of the lung, respectively.[59–61] The aberrant kinase activity of these ALK-fusion proteins is thought to be caused by transphosphorylation upon self-dimerization through their N-terminal domain derived from the fusion partners. Mutations or deletions of the dimerization domain of NPM–ALK and EML4–ALK result in loss of the transforming capacity of the fusion kinases.[55,57] The constitutive active fusion kinases transmit signals through activation of a variety of signal transducers, including PLCγ, PI3K/AKT, STAT3 and RAS.[62–67]

In neuroblastoma, on the other hand, aberrant activation of ALK kinase is caused by gene amplification[31] or mutations.[11–14] Thus, ALK represents a unique type of oncogenic kinase, in that it is deregulated either by gene fusions, or by gene amplification or mutations, depending on the tumor type.

Biological consequences of ALK mutations

Most reported *ALK* mutations occurred within the kinase domain, in which three highly conserved amino acid positions, F1174, F1245 and R1275, were predominantly affected, suggesting their functional importance for the regulation of kinase activity[11–14] (Figs 3,4). The F1174 residue is located at the end of the Cα1 helix and corresponds to equivalent positions mutated in EGFR (V769) and ERBB2 (V769), while the F1245 lies in the catalytic domain and corresponds to the L833 residue of EGFR, a mutation of which is reported to be associated with gefitinib resistance in lung cancer (Fig. 5).[13] The R1275 position lies within the activation loop and is invariably changed to glutamine, and amino acid substitution at this position to a positively charged one would displace the loop to positions that permit autophosphorylation and autoactivation of the kinase (Fig. 5).[68,69] However, the distributions of these mutations were different between sporadic cases and familial cases; R1275 mutations are commonly found in both sporadic and familial cases, while no germline mutations involving the F1174 or F1245 position have been reported.[11–14] Because not all mutant *ALK* carriers develop neuroblastoma (i.e. incomplete penetrance), a germline *ALK* mutation is not fully oncogenic and additional genetic events are thought to be required for neuroblastoma development. *ALK* mutations tend to be associated with advanced diseases and also with *MYCN* amplification in sporadic neuroblastoma cases, although the trend was not clear for germline *ALK* mutations.[11–14]

When expressed in NIH3T3 cells, the predominant kinase domain mutant (F1174L) and a juxtamembrane mutant (K1062M) are shown to have transforming capacity; mutant-transduced cells display increased colony formation in soft agar and tumor generation in nude mice, whereas the mutant kinases show increased autophosphorylation and *in vitro* kinase activity compared with wild-type ALK.[14] In addition, when introduced into an IL-3-dependent cell line, BaF3, the two major kinase domain mutants (F1174L and R1275Q), render the cell line independent of IL-3.[13] Expression of the F1174L mutant in NIH3T3 and Ba/F3 cells leads to constitutive activation of the downstream signaling pathways of the ALK kinase, as demonstrated by increased levels of phosphorylated ERK1/2, STAT3 and AKT.[13,14] These functional and biochemical studies together indicate that these ALK mutants are actually oncogenic and could be responsible for the pathogenesis of neuroblastoma.

doi: 10.1111/j.1349-7006.2010.01825.x

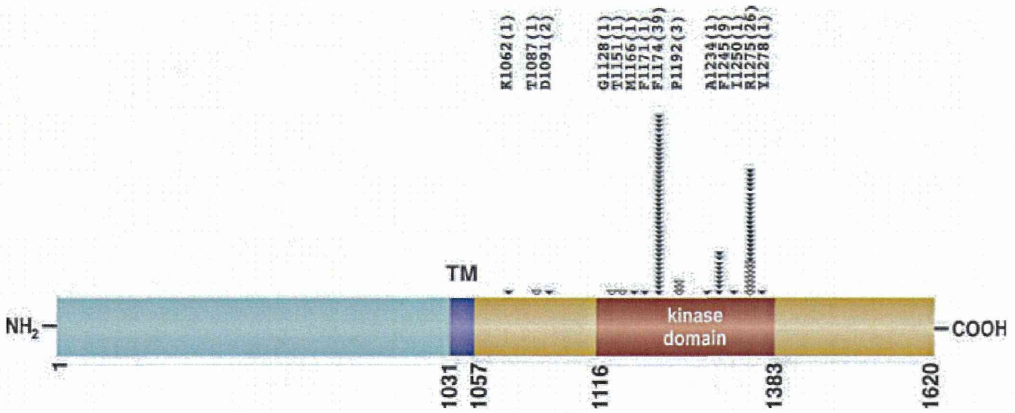

Fig. 3. Frequency and distribution of *ALK* mutations reported in familial and sporadic cases of neuroblastoma.[11–14,27] Locations of somatic and germline mutations of *ALK* in each case or family are depicted by filled and open arrows, respectively. The exact positions and amino acids involved are indicated on the top, where the number of reported mutations is indicated in parenthesis.

```
                    1174
HUMAN ALK     ...ALIISKFNHQNIVR..
HUMAN LTK     ...ALIISKFRHQNIVR..
HUMAN INSR    ...ASVMKGFTCHHVVR..
HUMAN IGF1R   ...ASVMKEFNCHHVVR..

                    1245
HUMAN ALK     ...EENHFIHRDIAARN..
HUMAN LTK     ...EENHFIHRDIAARN..
HUMAN INSR    ...NAKKFVHRDLAARN..
HUMAN IGF1R   ...NANKFVHRDLAARN..

                    1275
HUMAN ALK     ...GDFGMARDIYRASY..
HUMAN LTK     ...GDFGMARDIYRASY..
HUMAN INSR    ...GDFGMTRDIYETDY..
HUMAN IGF1R   ...GDFGMTRDIYETDY..
```

Fig. 4. Alignment of amino acids of ALK among different species. Conserved amino-acids among different insulin receptor family kinases are shown by gray boxes and the mutated positions are shown in red.

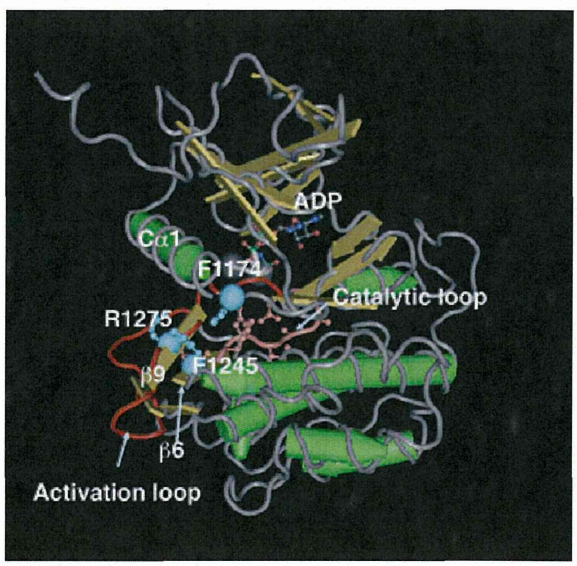

Fig. 5. A 3-D structure of the kinase domain of ALK kinase predicted from that solved for IGF-1R, where the positions of three major mutations are indicated by light blue spheres. Activation and catalytic loops are depicted by red and pink wires.

Effects of ALK inhibition on ALK fusion kinases

The critical role of *ALK* mutations in neuroblastoma development is further supported by the experiments using inhibition of mutant ALK. Tumor suppressive effects of ALK inhibition have been well documented in *NPM–ALK*-positive ALCL and *EML4–ALK*-positive NSCLC. NVP–TAE684 is a highly potent and selective small molecule ALK inhibitor, which blocks the growth of ALCL-derived cell lines with very low IC_{50} values between 2 and 10 nM.[70] NVP–TAE684 treatment of ALCL-derived cell lines induces rapid and sustained inhibition of phosphorylation of NPM–ALK and its downstream signaling, leading to cell cycle arrest and apoptosis.[70] NVP–TAE684 also induces varying degrees of growth suppression in *EML4–ALK*-bearing lung cancer cell lines, including NCI-H3112, NCI-H2228 and DFCI032.[67,71] PF-2341066 was another compound, which was initially identified as an orally available c-Met inhibitor in biochemical enzymatic screens, but was subsequently found to show selective inhibition of ALK.[72,73] It is highly selective for both ALK and c-Met kinases, being almost 20-fold selective for ALK and c-Met compared with 120 other kinases.[73] PF-2341066 inhibited cell growth of NPM–ALK-positive ALCL-derived cell lines, as well as EML4–ALK-positive NSCLC-derived cell lines with decreased downstream signaling pathways, although their IC_{50} values were significantly higher than those of NVP–TAE684.[71,72] Recently, Soda *et al.* generated transgenic mice, in which the *EML4–ALK*-transgene was selectively expressed in the developing lung under the *surfactant protein C* promoter.[61] All mice developed multiple lung adenocarcinomas soon after birth, which were successfully treated with a 2,4-pyrimidinediamine derivative that specifically inhibits ALK kinase.[61] These observations strongly support that aberrant ALK activity of ALK-fusion proteins is central to the development of ALCL and NSCLC.

Effects of ALK inhibition on *ALK*-mutated neuroblastoma cell lines

In neuroblastoma, the predominant mechanism of ALK activation should be some conformational change caused by a point mutation typically involving the kinase domain, which potentially affects the kinetics of ALK inhibitors on the mutated kinase. However, as long as major ALK mutants are concerned, their kinase activity seems to be successfully inhibited by the currently available ALK inhibitors. Ba/F3 cells transduced with the F1174L or R1275Q ALK mutant were effectively killed by NVP–TAE684 or PF-2341066, whereas the cells transduced with a constitutive active FLT3 mutant or wild-type ALK were not.[13] Thus, both compounds specifically inhibit the kinase activity of these ALK mutants, although the inhibition is more efficient for F1174F than for R1275Q. In fact, many, if not all, neuroblastoma cell lines carrying mutated or amplified *ALK* alleles are shown to be sensitive to these ALK inhibitors.[12,13,71] Interestingly, the sensitivity of some neuroblastoma cell lines to small molecule ALK inhibitors was recognized prior to the discovery of *ALK* mutations in neuroblastoma. McDermott *et al.* tested more than 600 cancer cell lines for their sensitivity to NVP-TAE684 and/or PF-2341066 and found that neuroblastoma cell lines, as well as cell lines derived from ALCL and lung cancer, frequently show sensitivity to these inhibitors.[71] The dependence of *ALK*-mutated neuroblastoma to ALK inhibition is further confirmed by ALK knockdown experiments; shRNA-mediated knockdown of ALK in *ALK*-mutated neuroblastoma cell lines results in the suppression of cell growth, indicating that the major effect of ALK inhibitors on *ALK*-mutated neuroblastoma cell lines are mediated by their activity on ALK rather than off-target effects on other kinases.

As mentioned above, the sensitivity of *ALK*-mutated neuroblastoma cell lines to ALK inhibitors seems to substantially differ among cell lines, depending on the type of *ALK* mutations. The F1174L mutant seems to be more sensitive to NVP–TAE684 than the R1275Q mutant.[13] Some *ALK*-mutated cell lines were resistant to ALK inhibition; SMS–KCNR harbors the R1275Q mutation, but was not killed by NVP–TAE684 or shRNA, indicating that this cell line acquired some additional mutations, escaping from its dependence on ALK signaling.

Concluding remarks

Genetic analyses of neuroblastoma have revealed that aberrant activation of ALK kinase in human cancer is not only caused by gene fusions but also by gene amplification or germline/somatic mutations. However, probably the most significant impact of the discovery of *ALK* mutations in neuroblastoma would be the possibility of successful treatment of *ALK*-mutated neuroblastoma with small molecule ALK-inhibitors, which are now under development in several pharmaceutical companies. Because ALK expression is restricted to developing neural tissues and ALK-deficient mice develop normally,[37] mutated ALK is likely to be a plausible therapeutic target. Although the enthusiasm for *ALK*-targeted therapy for advanced neuroblastoma seems to be too early at this moment, an encouraging result was reported from a clinical trial of crizotinib (PF-2341066) for NSCLC carrying the *EML4–ALK* fusion gene. A total of 50 patients were evaluable for response, where 64% of the overall response rate and 90% of the disease control rate were obtained[74] with minimum adverse reactions. Nevertheless, the result in NSCLC is not easily translated into neuroblastoma cases. For example, while some ALK mutants are shown to be inhibited by the available ALK inhibitors *in vitro*, the impact of different mutation types on the action of inhibitors should be further evaluated. The effect of frequent co-existence of *MYCN* amplification with *ALK* mutations on sensitivity to ALK inhibitors is still elusive, although a cell line, KELLY, which carries both the F1174L mutation and *MYCN* amplification, was reported to be sensitive to NVP–TAE684.[13,71] Finally, the role of ALK inhibitors in *ALK*-non-mutated neuroblastoma is another interest. Some neuroblastoma cell lines (NBEBC1 and NB1771) were shown to be sensitive to shRNA-mediated ALK knockdown, even though they were reported to have no mutated *ALK* alleles.[11] Interestingly, ALK is phosphorylated in these cell lines at lower levels. Considering the frequent expression of ALK in neuroblastoma cells, it may be postulated that regardless of its mutation status, ALK play a positive role during the initiation and promotion of neuroblastoma, even though established tumors may or may not depend on the ALK activity. Clearly, much more work is required before the clinical role of ALK inhibitors in the treatment of advanced neuroblastoma is established.

Disclosure Statement

The authors have no conflict of interest.

References

1 Maris JM, Hogarty MD, Bagatell R, Cohn SL. Neuroblastoma. *Lancet* 2007; **369**: 2106–20.
2 Hiyama E, Iehara T, Sugimoto T *et al.* Effectiveness of screening for neuroblastoma at 6 months of age: a retrospective population-based cohort study. *Lancet* 2008; **371**: 1173–80.
3 Brodeur GM. Neuroblastoma: biological insights into a clinical enigma. *Nat Rev Cancer* 2003; **3**: 203–16.
4 Pearson AD, Pinkerton CR, Lewis IJ, Imeson J, Ellershaw C, Machin D. High-dose rapid and standard induction chemotherapy for patients aged over 1 year with stage 4 neuroblastoma: a randomised trial. *Lancet Oncol* 2008; **9**: 247–56.
5 De Bernardi B, Nicolas B, Boni L *et al.* Disseminated neuroblastoma in children older than one year at diagnosis: comparable results with three consecutive high-dose protocols adopted by the Italian Co-Operative Group for Neuroblastoma. *J Clin Oncol* 2003; **21**: 1592–601.
6 Matthay KK, Villablanca JG, Seeger RC *et al.* Treatment of high-risk neuroblastoma with intensive chemotherapy, radiotherapy, autologous bone marrow transplantation, and 13-cis-retinoic acid. Children's Cancer Group. *N Engl J Med* 1999; **341**: 1165–73.
7 Brodeur GM, Seeger RC, Schwab M, Varmus HE, Bishop JM. Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage. *Science* 1984; **224**: 1121–4.
8 Seeger RC, Brodeur GM, Sather H *et al.* Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastomas. *N Engl J Med* 1985; **313**: 1111–6.
9 Katzenstein HM, Bowman LC, Brodeur GM *et al.* Prognostic significance of age, MYCN oncogene amplification, tumor cell ploidy, and histology in 110 infants with stage D(S) neuroblastoma: the pediatric oncology group experience – a pediatric oncology group study. *J Clin Oncol* 1998; **16**: 2007–17.
10 Hansford LM, Thomas WD, Keating JM *et al.* Mechanisms of embryonal tumor initiation: distinct roles for MycN expression and MYCN amplification. *Proc Natl Acad Sci U S A* 2004; **101**: 12664–9.
11 Mosse YP, Laudenslager M, Longo L *et al.* Identification of ALK as a major familial neuroblastoma predisposition gene. *Nature* 2008; **455**: 930–5.
12 Janoueix-Lerosey I, Lequin D, Brugieres L *et al.* Somatic and germline activating mutations of the ALK kinase receptor in neuroblastoma. *Nature* 2008; **455**: 967–70.
13 George RE, Sanda T, Hanna M *et al.* Activating mutations in ALK provide a therapeutic target in neuroblastoma. *Nature* 2008; **455**: 975–8.

doi: 10.1111/j.1349-7006.2010.01825.x