Appearance of the novel activating F1174S ALK mutation in neuroblastoma correlates with aggressive tumor progression and unresponsiveness to therapy

Tommy Martinsson¹, Therese Eriksson², Jonas Abrahamsson³, Helena Caren¹, Magnus Hansson⁴, Per Kogner⁵, Sattu Kamaraj², Christina Schönherr², Joel Weinmar², Kristina Ruuth², Ruth Palmer², Bengt Hallberg²

Departments of ¹Clinical Genetics, ³Pediatrics and ⁴Pathology, The Sahlgrenska Academy, University of Gothenburg, S-41345 Gothenburg, Sweden. ⁵Childhood Cancer Research Unit, Department of Womens and Childrens Health, Karolinska Institutet, Stockholm, Sweden. ²Department of Molecular Biology, Building 6L, Umeå University, Umeå 901 87, Sweden.

Précis:
Mutation of a critical kinase in neuroblastoma progression may be missed in the initial tumor biopsy requiring testing later at progression.

Running title: ALK F1174S mutation acquired during NB progression

Prepared as a research article for Cancer Research

*Correspondence: Prof. T Martinsson; E-mail: tommy.martinsson@gu.se,
Phone: +46 31 343 4803, Fax: +46 31 842160
Abstract

Mutations in the kinase domain of the ALK kinase have emerged recently as important players in the genetics of the childhood tumor neuroblastoma. Here we report the appearance of a novel ALK mutation in neuroblastoma, correlating with aggressive tumor behaviour. Analyses of genomic DNA from biopsy samples initially showed ALK sequence to be wild type. However, during disease progression mutation of amino acid F1174 to a serine within the ALK kinase domain was observed, which correlated with aggressive neuroblastoma progression in the patient. We show that mutation of F1174 to serine generates a potent gain-of-function mutant, as observed in two independent systems. Firstly, PC12 cell lines expressing ALK^{F1174S} display ligand independent activation of ALK and further downstream signaling activation. Secondly, analysis of ALK^{F1174S} in Drosophila models confirms that the mutation mediates a strong rough eye phenotype upon expression in the developing eye. Thus, we report a novel ALK^{F1174S} mutation, which displays ligand independent activity in vivo, correlating with rapid and treatment resistant tumor growth. The study also shows that initial screening in the first tumor biopsy of a patient may not be sufficient and that further molecular analyses in particular in tumor progression and/or tumor relapse is warranted for better understanding of the treatment of neuroblastoma patients.
Introduction

The ALK gene (OMIM: 105590) was initially characterized as a fusion partner of the NPM-ALK oncogene and is now recognized as the active component in multiple fusion proteins in a variety of cancers (1). Recent studies have reported putative activating ALK point mutations in both familial and sporadic cases of neuroblastoma, and in a number of neuroblastoma cell lines (2-6). Neuroblastoma, which is derived from neural crest cells and can occur in the entire peripheral sympathetic nervous system, accounts for approximately 15% of all deaths in pediatric cancer (7).

In this study we have identified a novel activating mutation in ALK in a neuroblastoma patient in the course of analysis of genomic DNA from patient biopsy samples. Initial analysis indicated a wild type ALK locus, which during disease progression, became mutated leading to the production of a F1174 ALK kinase domain mutation. In inducible cell lines expressing the F1174 ALK mutant as well as in the Drosophila model system we are able to show that the F1174 ALK variant is potently activated in the absence of ligand stimulation leading to the activation of downstream signaling events. The appearance of this novel F1174S ALK mutant correlates with the development of aggressive disease at the patient level with emergence of therapy resistance and fatal outcome.
Materials and methods

Patient

On a routine check at the child welfare centre an 11 ½ months old boy was found to have an asymptomatic left sided abdominal mass. CT scan demonstrated a cystic multiseptal tumor at the place of the left adrenal gland. MIBG scintigraphy showed uptake in the tumor capsule and in three sites in the skeleton (right femur and tibia, left forearm). Urinary catecholamine metabolites and serum neuron specific enolase were slightly elevated. Bone marrow biopsy showed no tumor cells. A biopsy of the primary tumor showed undifferentiated neuroblastoma without amplification of MYCN. According to the INRG classification (8) the tumor would have been classified as intermediate risk.

The boy was treated according to the European Infant Neuroblastoma Study Protocol for stage IV (trial 99.3). He first received two courses of etoposide and carboplatin followed by four courses of CADO (cyclophosphamide, doxorubicin, vincristine). Evaluation with MIBG and CT scan after two, four and six courses demonstrated a steady decrease in size of the primary tumor but the MIBG uptake in the right tibia persisted and CT scan showed suspected tumor growth (Fig. 1A). The adrenal tumor was radically resected and at the same time a biopsy from the tibial bone demonstrated viable tumor cells. He proceeded to chemotherapy with OJEC (vincristine, carboplatin, etoposide and cyclophosphamide) but within a week he rapidly developed an increasingly swollen left lower leg and CT scan showed progressive destruction of the bone with a large soft tissue component and several new metastases in the groin (Fig. 1B). Due to the extremely rapid growth a new biopsy including tumor genetic investigations was performed. A further course of TVD (topotecan, vincristine, doxorubicin) was given but the tumors progressed and he developed lung metastases and died six weeks after the last course, ten months after initial diagnosis.

Immunohistochemistry

Staining for Ki-67 (DAKO), NB84 (Novocastra), ALK-P (Abcam) and ALK (Abcam) was made using the DAKO Autostainer. The slides were rehydrated with Xylene followed by a series of alcohol dilutions and a rinse in buffer (DAKO 8007). PTLINK (DAKO) was used as
enzyme antigen retrieval method. Endogenous enzyme block was made during 5 minutes with EnVision FLEX Peroxidase-Blocking Reagent (DAKO). The antibodies (diluted at DAKO Company) were incubated at room temperature for 20 minutes. After a 5 minutes rinse in buffer the labelled polymer, EnVision FLEX/HRP (DAKO), was applied and incubated for 20 minutes with two additional rinses before the slides were incubated with substrate-chromogen, Substrate Working Solution (DAKO) for 10 minutes. The slides were ultimately rinsed and counterstained in EnVision FLEX Hematoxylin and mounted.

Genomics profile with SNP array

Microarray analyses of DNA from the tumor samples were performed using Affymetrix human 250K gene mapping arrays as described earlier (9, 10). For primary data analysis the GDAS software (Affymetrix) was used, while genomic profiles were generated using CNAG (Copy Number Analyzer for Affymetrix GeneChip Mapping arrays) version 3.0 (Genome Laboratory, Tokyo University; http://wwwgenome.umin.jp; 11).

Detection of ALK mutation with DNA sequencing

The procedure for DNA sequencing and analysis of the ALK exons has been described earlier (2).

Generation of human ALK mutant constructs in PC12 cells.

Human ALK full length cDNA in the pcDNA3 vector was a kind gift from Marc Vigny. A 1298 bp fragment corresponding to nucleotides 4061 to 5358 of wild type human ALK sequence (NCBI Reference Sequence: NM_004304.3) was PCR amplified using the following primers: 5’-TTCTCCGGCATCATGATTGTGTA -3’ and 5’- TTGGACTGAGAGAATGCCATATT-3’. This PCR product was subcloned into pCRII-TOPO (Invitrogen, following manufacturer’s instructions), and the resulting plasmid was used as template for generation of the 3521T→C point mutation, corresponding to the F1174S mutation, utilizing Quick Change Site-Directed mutagenesis kit (Stratagene, according to the manufacturer’s instructions) with the following primers: 5’- GGAAGCCCTGATCATCAGCAAATCCAACCAGAAC-3’ and 5’-
GTTCTGGTGTTGATTTGCTGATGATCAGGGCTTCC-3’. The F1174L mutation (3522C→A) was generated with the following primers: 5’-
GGAAGCCTGATCATCAGCAAAATTAAACCACCAAACA-3’ and 5’-
TGTTCTGGGTGTTAATTGCTGATCAGGGGCTTCC-3’. The generated 3521T→C and 3522C→A point mutations were confirmed by sequencing from both directions. The mutated ALK fragment was digested with BlpI-FseI restriction enzymes and ligated into the BlpI-FseI sites (position 4143 and 5309) of the full length ALK in pcDNA3. Thereafter, both pcDNA3 wild type and ALK\textsuperscript{F1174S} were cut with EcoRI and NotI and transferred to the inducible cell expression vector pTTP (12).

**Cell culture**

Stable PC12 Tet-on clones expressing human pTTP-ALK and pTTP-ALK\textsuperscript{F1174S} were generated by transfecting (Lipofectamine, Invitrogen) PC12 Tet-on cells according to manufacturer’s instructions (BD Biosciences, cat. # 630921). Stable clones were selected in Dulbecco modified Eagle medium, containing 10% horse serum (MP biomedicals, Ohio, USA), 5% tetracycline-screened fetal calf serum (HyClone, Utah, USA), penicillin, streptomycin, L-glutamine, 100 µg/ml G418 and 2 µg/ml puromycin at 37 °C and 5% CO\textsubscript{2}. Cell clones were screened for ALK expression by induction with 1 µg/ml doxycycline for 24 hours prior to analysis by immunoblotting.

**Neurite formation assay**

PC12-cells 2x10\textsuperscript{6} were transfected by electroporation, in a Amaxa electroporator, using 1.5 µg pCDNA3-hALK and 0.5 µg pEGFPN1 (Clontech, Mountain View, CA, USA) and 100 µl Ingenio electroporation solution (Mirrus Bio LCC, Madison, WI, USA). After transfection cells were transferred to DMEM supplemented with 7% horse serum and 3% FBS and seeded into 24-well plates, mAb31 was added at 1 µg/ml. 2 days after transfection the fraction of GFP-positive and neurite carrying cells versus GFP positive cells was estimated under a Zeiss Axiovert 40 CFL microscope. To be judge as a neurite carrying cell the neurites of the cell had to reach at least the length of 2x the diameter of a normal cell.
Transformation assay:

NIH3T3 cells 45x10^3/well were seeded the day before transfection in 12 well plates (Nunc, Roskilde, Denmark) coated with collagen, PureCol (Advanced Biomatrix, San Diego, CA, USA). The cells were transfected with 0.55 µg pCDNA3 or pCDNA3-hALK and 1.4 µl Lipfectamine2000 (Invitrogen, Paisley, UK) in 0.3 ml OptiMEM (Invitrogen), after 6 hours the transfection was interrupted by adding 2 ml DMEM with 10% heat inactivated FBS to the wells. After 24h, 3/5 of the cells from each well were transferred to 6-well plates (Nunc), coated with collagen. Cells were maintained in DMEM, 10% heat inactivated FBS and 0.5 mg/ml G418 (Invivogen, San Diego, CA, USA) until they reached confluence after 12 days, with replacement of medium each third day. Thereafter the cells were kept in DMEM with 5% heat inactivated FBS and 0,25mg/ml G418 for another 10 days, with replacement of medium each second day. Plates were washed with PBS and air dried, fixed with methanol for 20 min and stained with 0.2% crystal violet in 20% ethanol for 20 min followed by a short rinse in water. The number of foci was determined by inspection under the naked eye.

Cell lysis, immunoprecipitation and immunoblotting

Cells were washed twice with ice cold PBS prior to harvest in lysis buffer (25mM Tris pH 7.5, 150 mM NaCl, 1% [vol/vol] Triton X-100, 1 mM DTT, 1 mM EDTA, protease inhibitor tablet (Roche, Mannheim, Germany). Cell lysates were cleared by centrifugation at 12 000 rpm for 15 min at 4 °C. For immunoprecipitations, cell lysates were incubated with 4 µg antibodies for one hour at 4 °C, followed by incubation with 30 µl Protein G beads for 30 minutes at 4 °C. Beads were washed once in lysis buffer, followed by five times in wash buffer (25mM Tris pH 7.5, 300 mM NaCl, 1% [vol/vol] Triton X-100, 1 mM DTT). Samples were boiled in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and analyzed by immunoblotting. Samples were boiled in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and analyzed by immunoblotting.
Generation of human ALK mutant constructs in *Drosophila melanogaster.*

Prior to ligation of ALK<sup>F1174L</sup> and ALK<sup>F1174S</sup> from pcDNA3 into the *Drosophila* pUAST expression vector, a 898 bp fragment preceeding the translation start was removed to increase expression efficiency in *Drosophila* (details available upon request). All three constructs were subsequently subcloned into the EcoRI-NotI site of the pUAST *Drosophila* vector and the resulting constructs were verified by DNA sequencing analysis. Transgenic constructs were employed for the generation of transgenic fly strains (BestGene Inc, CA).

Fly strains and expression experiments.

Standard *Drosophila* husbandry procedures were followed. The following stocks were used: *w<sup>1118</sup>* (Bloomington, stock number 5905) and pGMR-Gal4 (Bloomington, stock number 9146). The trangenic fly strains *UAS-ALK<sup>wt</sup>, UAS-ALK<sup>F1174</sup>, and UAS-ALK<sup>F1174S</sup> were generated as described above. ALK proteins were specifically expressed in the eye tissue of *Drosophila* by crossing the *UAS-ALK* transgenic flies to the pGMR-GAL4 driver. Fly crosses were kept at 25 °C. Eye discs from 3rd instar larvae were fixed in 4% formaldehyde, blocked in 5% NGS overnight at 4°C, and stained with primary antibody anti-ALK mAb46 (13) and secondary antibody anti-mouse Cy3 (The Jackson Laboratory, Bar Harbor, ME). Fluorescent microscopy pictures of eye discs were taken on a Leica TCS SPE confocal microscope. Electron microscopy pictures of adult fly eyes were taken on a Cambridge Stereoscan 360 iXP scanning electron microscope at the Electron Microscopy Platform at Umeå University.
Results and Discussion

The patient presented with an abdominal tumor mass and skeletal involvement without bone marrow involvement, at 11 months of age. Biopsy of the primary tumor at diagnosis showed undifferentiated neuroblastoma without amplification of MYCN. The SNP array genomic profiling performed at diagnosis (Fig 1A) did not display any genomic aberration in the ALK gene region. According to the International Neuroblastoma Risk Group Classification (INRG; 8) the tumor was intermediate risk, and the patient was treated according to the European Infant NB Study Protocol for MYCN-nonamplified stage 4. Evaluation with iodine-123 metaiodobenzylguanidine scintigraphy (MIBG) and computerized tomography (CT) at different dates showed an initial decrease in size of the primary tumor but persistent metastatic tumor in right tibia (Panel E). Almost seven months after diagnosis, the primary adrenal tumor was radically resected but simultaneous tibial bone biopsy demonstrated viable tumor cells. At this time, immunohistochemistry for ALK-P showed a very weak and heterogeneous staining, identical to that in the initial biopsy in both the primary and tibial tumor (figure 1E). The patient received vincristine, etoposide and carboplatin but within a week developed progressive destruction of the bone with a large soft tissue component and several new metastases in the groin (Panel F). Due to the extremely rapid growth, a new biopsy with reinvestigation of tumor genetics was performed – now eight months after diagnosis. At this time, a new copy-neutral LOH of a part of chromosome 2p (including the locus for the ALK gene) was detected as the sole new feature of the array profile (Panel A, black arrow), as compared to the profile at diagnosis. A course of topotecan, vincristine and doxorubicin was administered but the disease progressed and the patient developed lung metastases and died six weeks after the last course, ten months after initial diagnosis. DNA sequence analysis of the ALK gene of the second tumor sample revealed a homozygous mutation of ALK exon 23 mutation 3521T>C leading to homozygosity for missense mutation F1174S (Panel D). ALK amino acid position F1174 is one of the mutation hotspots in neuroblastomas displaying ALK mutation (2-6).

Thus, the very rapid progress of the tumor and development of refractoriness to chemotherapy coincided with the acquirement of a homozygous ALK tyrosine kinase domain
mutation not present in the germline DNA of the patient. It is tempting to speculate on a two-step genetic process in which (i) the ALK\textsuperscript{F1174S} mutation arises on one of the two chromosome 2 copies in the tumor and that (ii) this copy is duplicated through a homologous recombination event with full retained heterozygosity for the proximal 2p and the whole 2q arm. Thus, the primary tumor biopsy and the sample from the last biopsy were similar in that the two aberrations found at diagnosis (10q loss and 17q gain) were found also in the latter sample, but strikingly the latter sample differed in that it had gained an additional aberration, a homozygotization of a large portion of chromosome region 2p including a novel homozygous F1174S mutation in ALK. This is the first described case of an ALK mutation shown to be acquired during the disease course, concomitant with a tumor progression “from bad to worse”.

To investigate whether the ALK\textsuperscript{F1174S} mutation was a major cause for the dramatic tumor progression we employed both \textit{Drosophila} melanogaster and cell culture based systems to examine the nature of ALK\textsuperscript{F1174S}. \textit{Drosophila melanogaster} offers a number of advantages including the conservation of many molecular pathways with vertebrates. The \textit{Drosophila} ALK receptor tyrosine kinase (RTK) mediates activation of the ERK pathway in the developing visceral mesoderm (14-17), a signaling pathway which is crucial for the formation of the fly gut \textit{in vivo}. Ectopic expression of human UAS-ALK in the \textit{Drosophila} eye employing the pGMR-Gal4 driver line, which directs protein expression in the developing photoreceptors of the eye, does not result in any obvious phenotype in adult flies (Fig 3B,F), and is similar to wild type controls (Fig 3A,E). Expression of the various human ALK proteins confirmed by immunohistochemical analysis of developing eye discs (Fig 3I, J and K). Thus, the wild type ligand dependant human ALK RTK does not appear to be activated by endogenous \textit{Drosophila} ligands, providing a clean background in which to analyse the activating potential of putative activating mutants of the human ALK as identified in neuroblastoma patients.

Given the clean phenotypic background observed with overexpression of the wild type human ALK, we proceeded to investigate the \textit{in vivo} signaling potential of the putative activated ALK\textsuperscript{F1174S} mutant in the \textit{Drosophila} system. In contrast to the results obtained with overexpression of wild type human ALK, we observed a robust ligand independent signaling upon ectopic expression of human ALK\textsuperscript{F1174S} leading to the destruction of normal tissue.
morphology in the developing fly eye (Fig 3C, G). The level of ligand independent signaling induced by ALK$^{F_{1174S}}$ was comparable with that observed upon ectopic expression of human ALK$^{F_{1174L}}$ (Fig 3D, H), a previously verified ALK gain-of-function mutation (3). The destructive effects of ectopic expression of human ALK$^{F_{1174S}}$ and ALK$^{F_{1174L}}$ in the fly eye could already be observed during third instar larval stages, where the definition of ommatidial units in the developing fly eye were destroyed (Fig 3M, N) as compared with expression of wild type ALK (Fig 3L). Since no human ALK ligand is present during the development of the Drosophila eye, as evidenced by the lack of phenotype observed upon expression of wild type human ALK, these results confirm that the human ALK$^{F_{1174S}}$ mutant is indeed a ligand independent activating mutant of the ALK RTK in vivo.

To further evaluate the ALK$^{F_{1174S}}$ mutant we employed a number of different cell culture based systems. Initially, we developed an inducible PC12 cell culture system for clonal expression of wild type and the ALK$^{F_{1174S}}$ mutants. PC12 cells were induced to express either wild type ALK or ALK$^{F_{1174S}}$ by addition of tetracycline (Fig 4A). Wild type ALK can be activated with an agonist monoclonal antibody (mAb31) leading to extensive tyrosine phosphorylation of the receptor and activation of downstream targets, such as Erk (Fig 4A, compare lane 3 with 2). Induction of expression of ALK$^{F_{1174S}}$ leads to transphosphorylation of the mutant receptor in a ligand independent manner, in agreement with our observations in the Drosophila model system. The autophosphorylated human ALK$^{F_{1174S}}$ mutant is able to activate the downstream target Erk in a ligand independent manner (Fig 4A), and did not appear to be further activated upon mAb31 stimulation (Fig 4A, compare lanes 8 and 6 with 5). Addition of TAE684, a small molecule inhibitor of ALK, completely abolished the activation of Erk observed with both the mAb31-activated wild type ALK and the ALK$^{F_{1174S}}$ mutant (18) (Fig 4A, compare lanes 9 and 4 with 8 and 3, respectively). In parallel we also compared the phosphorylation status of ALK$^{F_{1174S}}$ with a known gain-of-function mutation of ALK, the ALK$^{F_{1174L}}$ (3). Transiently transfected ALK$^{F_{1174S}}$ and ALK$^{F_{1174L}}$ exhibit similar phosphorylation status and activation of the Erk pathway (Supplementary figure 1). Thus, it is clear that the ALK$^{F_{1174S}}$ mutant displays gain-of-function activity, providing an explanation for the devastating progression of this patient’s neuroblastoma.

Secondly, we investigated whether the gain of function ALKF1174S mutant was capable of stimulating neurite outgrowth. We and others have previously shown that activation of ALK
triggers differentiation of PC12 cells into sympathetic-like neurons, a process characterized by extension of neurites (19-22). Expression of both wild type ALK and the ALK_F1174S mutant was induced in PC12 cells by addition of doxycyclin (Fig. 4B). The activating antibody mAb31 was added simultaneously to cells expressing wild type ALK. Images were acquired 48h after incubation in presence of doxycyclin and mAb31. Expression of either ALK_F1174S or ALK_F1174L mutants mediates clear neurite outgrowth within 48hrs, even in the absence of activating monoclonal antibody mAb31 (Fig. 4B). Importantly, expression of ALK in the absence of activating antibodies mediates only a minor fraction of neurite outgrowth (Fig. 4B). However, upon stimulation of wild type ALK with mAb31 a similar level of neurite outgrowth to that of the F1174S and F1174L mutants is observed (Fig. 4B). Thus, in inducible cell lines expressing the ALK_F1174S mutant as well as in the Drosophila model system we are able to show that the ALK_F1174S variant is potently activated in the absence of ligand stimulation leading to the activation of downstream signaling events. Finally, we investigated whether the human ALK_F1174S displays transforming potential. NIH3T3 cells were transfected with human ALK_F1174S. Expression of human ALK_F1174S and ALK_F1174L mediates formation of foci of transformed cells over the background monolayer (Fig. 4C), whereas expression of the wild type human ALK receptor is unable to mediate foci formation. Thus, the human ALK_F1174S shows intrinsic transforming activity.

Taken together, the results presented here clearly demonstrate that the appearance of the novel ALK_F1174S mutant correlates with the development of aggressive neuroblastoma in this patient. Our results with the small molecular ALK inhibitor TAE684 suggest that the development of small molecular inhibitors specific for ALK, such as PF02341066, may benefit and increase the possibility of treatment of neuroblastoma patients. The study also clearly shows that initial screening in the first tumor biopsy of a patient is not be sufficient and that further molecular analyses of the ALK locus in particular in tumor progression and/or tumor relapse is warranted for better understanding of the treatment of neuroblastoma patients.
Acknowledgements:

This work has been supported by grants from the Swedish Cancer Society (TM 06/1628 and 09/1217; BH 08-0597), the Children’s Cancer Foundation (TM 07/098; BH 08/084; RHP 08/074; SK 09/002), the Swedish Research Council (RHP 621-2003-3399), Association for International Cancer Research (RHP 08-0177), the Nilsson-Ehle foundation, the Assar Gabrielsson Foundation, the Wilhelm and Martina Lundgren Research Foundation and the Sahlgrenska University Hospital Foundation. RHP is a Swedish Cancer Foundation Research Fellow. H.C. is a recipient of a fellowship from the Swedish Knowledge Foundation through the Industrial PhD program in Medical Bioinformatics at the Strategy and Development Office (SDO) at Karolinska Institutet.
References


but is unable to activate the mouse ALK RTK. J Exp Zool B Mol Dev Evol 2007;308:269-82.
Legends for figures

Figure 1. Radiological and immunohistochemical findings in the neuroblastoma patient. A. Left upper radiograph shows status at end of induction. B. Right upper picture shows extensive progression of both soft tissue and skeletal lesions five weeks later despite chemotherapy. C-D. Immunohistochemistry findings in a needle biopsy at diagnosis using the neuroblastoma cell marker NB84 (C), and proliferation marker Ki-67 (D). E. The expression of Ki-67, in a biopsy taken nine months after diagnosis, is extremely high. F-G. The magnitude of ALK-P expression nine month after diagnosis (G) is increased compared to expression at diagnosis (F). H. No specific staining against ALK was found neither at diagnosis nor nine months after.

Figure 2. Molecular analyses of tumor material from neuroblastoma patient. A-B. SNP array genomic profile showing in the upper panels in both A and B gene dose data for all chromosomal regions, while the lower panels show allele dosage data for heterozygous loci (red - allele with stronger signal; green – allele with weaker signal. A. Genomic profile from tumor derived at diagnosis with 10q deletion and 17q gain. B. Genomic profile from tumor derived eight months after diagnosis with the previous aberrations plus a copy-neutral LOH event in chromosome region 2p (indicated with double arrow). C-D. Enlargement of chromosome 2 from the two biopsies, detailing the copy-neutral LOH event in 2p (D), and the relative positions of critical genes MYCN and ALK relative to the CN-LOH region. As can be seen in Figure 2C and 2D the MYCN gene region was not subjected to gene amplification or gain in this tumor, neither in the primary sample nor in the latter sample. E-G DNA sequencing of the ALK gene in the two tumor biopsy samples show homozygous missense mutation ALK exon 23 3521T>C; F1174S in the sample derived from the later biopsy (E), while the sample derived at diagnosis (F) and a control sample (G) display the normal 3521T/3521T genotype.

Figure 3. ALK\textsuperscript{F1174S} is a ligand independent activating mutation of ALK.
Ectopic expression of human ALKF_{1174}^S in the *Drosophila* eye results in a rough eye phenotype. A. Wild type *Drosophila* eye displaying a highly organized pattern of ommatidia. B-D, F-H. Ectopic expression of human wild type ALK, ALKF_{1174}^L and ALKF_{1174}^S employing the pGMR-GAL4 driver fly strain. B. Ectopic expression of wild type human ALK in the adult eye does not disrupt the wild type organized pattern of ommatidia, indicating that the receptor is inactive. C. Expression of human ALKF_{1174}^S in the adult eye causes a severe rough eye phenotype, suggesting that the F1174S mutation is a constitutively active form of the ALK receptor. D. Expression of human ALKF_{1174}^L in the fly eye also induces a rough eye phenotype. E. Close up of wild type eye in A. F. Close up of wild type ALK expressing eye in B. G. Close up of ALKF_{1174}^S induced phenotype in C. H. Close up of the ALKF_{1174}^L induced phenotype in D. I-K. Human ALK protein can be detected at the *Drosophila* larval stage in the eye discs with an anti-ALK antibody both after expression of wild type ALK (I), ALKF_{1174}^S (J) and ALKF_{1174}^L (K). L. Close up of the eye disc in I shows well organized pattern of photoreceptors that give rise to the organized pattern of ommatidia in the adult fly. Expression of wild type ALK does not affect the well organized photoreceptor pattern in the eye disc, suggesting that this form of the receptor is inactive. M. Close up of the eye disc in J shows that expression of ALKF_{1174}^S in this tissue disrupts the photoreceptor organization, indicating that the ALKF_{1174}^S mutation is constitutively active. N. Close up of the eye disc in K shows that the ALKF_{1174}^L expression also disrupts the photoreceptor pattern.

**Figure 4.** ALKF_{1174}^S mediates ligand independent phosphorylation and activation of Erk. A Tet-on inducible system for the expression of hALK and hALKF_{1174}^S was generated. PC12 cells were transfected with either wild type ALK or ALKF_{1174}^S, and inducible stable clonal cell lines were selected (see Material and Methods). Protein expression was induced with 1 µg/ml doxycylin (+) and serum starved for 24 hours prior to stimulation with 1µg/ml mAb31, an ALK activating monoclonal antibody for 30 minutes. Samples were analysed on SDS/PAGE, following by immunblotting with antibodies as indicated. Lanes 1 and 5: uninduced, lanes 2-4 and 6-9: induced expression of ALK and ALKF_{1174}^S, respectively. To observe the autophosphorylation status of expressed and induced ALK and ALKF_{1174}^S, cells were immunprecipitated using mAb4G10 prior to analysis on SDS/PAGE, followed by immunoblotting with anti-ALK antibody. Pan-Erk antibody was employed as loading control.
B. Transient transfection of hALK$^{F1174S}$ and hALK$^{F1174L}$ leads to neurite outgrowth 48 hours post induction. Cells transfected with wild type hALK were stimulated by the continuous presence of 1 µg/ml mAb31. Bars represent average percentage of neurite carrying and GFP positive cells versus GFP positive cells, from two independent transfections. C. ALK$^{F1174S}$ and ALK$^{F1174L}$ show similar transforming potential in NIH3T3 cells. Lower panel, data shown is the average of two independent experiments run in triplicates.
Fig. 1
**A** Tumor sample – at diagnosis

**B** Tumor sample – eight months after diagnosis

**C** Chromosome 2 – at diagnosis

**D** Chromosome 2 – eight months after diagnosis

**E** Homozygous mutation in ALK exon 23 3521T>C F1174S eight months after diagnosis

**F** Homozygous for the normal allele 3521T/3521T at diagnosis

**G** Homozygous for the normal allele 3521T/3521T control sample

*Copy neutral LOH for 0-38Mb part of chromosome region 2p*

Fig. 2

---

Break in position 38 Mb
ALK (position 30 Mb)
MYCN (position 16 Mb)
<table>
<thead>
<tr>
<th></th>
<th>wild type ALK</th>
<th>ALK (F1174S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>doxycyclin</td>
<td>- + + +</td>
<td>- + + + +</td>
</tr>
<tr>
<td>stim. m.Ab.31</td>
<td>- - + +</td>
<td>- - + + +</td>
</tr>
<tr>
<td>TAE684</td>
<td>- - - +</td>
<td>- - - + +</td>
</tr>
</tbody>
</table>

**A**

**B**

![Graph showing percentage of cells with neurites](image)

**C**

![Images of hALK-F1174L, hALK-F1174S, hALK-WT, pCDNA3](image)

**Fig. 4**

Downloaded from cancerres.aacrjournals.org on July 25, 2017. © 2010 American Association for Cancer Research.
Appearance of the novel activating F1174S ALK mutation in neuroblastoma correlates with aggressive tumour progression and unresponsiveness to therapy

Tommy Martinsson, Therese Eriksson, Jonas Abrahamsson, et al.

Cancer Res Published OnlineFirst November 8, 2010.