Open access • Journal Article • DOI:10.1042/BJ20081834

High incidence of DNA mutations and gene amplifications of the ALK gene in advanced sporadic neuroblastoma tumours. — Source link

Helena Carén, Frida Abel, Frida Abel, Per Kogner ...+1 more authors

Institutions: Sahlgrenska University Hospital, Chalmers University of Technology, Karolinska Institutet

Published on: 01 Dec 2008 - Biochemical Journal (Portland Press Limited)

Topics: Anaplastic lymphoma kinase, Gene duplication and Carcinogenesis

High incidence of DNA mutations and gene amplifications of the ALK gene in advanced sporadic neuroblastoma tumours

Helena Carén, Frida Abel, Per Kogner, Tommy Martinsson

High incidence of DNA mutations and gene amplifications of the *ALK* gene in advanced sporadic neuroblastoma tumours

Helena Carén[1], Frida Abel[1,2], Per Kogner[3] and Tommy Martinsson[1*]

[1]Department of Clinical Genetics, Institute of Biomedicine, University of Gothenburg, Sahlgrenska University Hospital, SE-41345 Göteborg, Sweden
[2] Department of Mathematical Statistics, Chalmers University of Technology, SE-412 96 Göteborg, Sweden
[3]Childhood Cancer Research Unit, Department of Woman and Child Health, Karolinska Institutet, Karolinska Hospital, SE-17176 Stockholm, Sweden

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

Running title: *ALK* alterations in neuroblastoma

Keywords: ALK, neuroblastoma, mutation, gene amplification, gene expression

ABSTRACT

Anaplastic lymphoma kinase (ALK) is oncogenic in several tumours and recently identified as a predisposition gene for familial neuroblastoma (NB) harbouring mutations in the tyrosine kinase domain (TKD). We have analysed a large set of sporadic human NB primary tumours of all clinical stages for chromosomal re-arrangements using array-CGH (n=108) and mutations of the *ALK* gene (n=90), and expression of *ALK* and related genes (n=19). *ALK* amplification or in-gene re-arrangements were found in 5% of NB tumours and mutations in 11% including two novel not previously published mutations in the TKD; c.3733T>A and c.3735C>A. DNA mutations in the TKD and gene amplifications were only found in advanced large primaries or metastatic tumours and correlated with the expression levels of *ALK* and downstream genes as well as other unfavourable features and poor outcome. Our data support that the ALK protein contribute to NB oncogenesis providing a highly interesting putative therapeutic target in a subset of unfavourable NB tumours.

INTRODUCTION

Neuroblastoma, the most common solid tumour of childhood, is an embryonal tumour of the postganglionic sympathetic nervous system. The most common genetic features of NB are amplification of the proto-oncogene *MYCN*, deletions of parts of chromosome arms 1p and 11q, gain of parts of 17q and triploidy. *MYCN* amplification, 1p loss and 17q gain are strongly associated with aggressive tumours and a poor outcome for the patient, whereas triploidy is associated with low-stage neuroblastomas with a good outcome [1-3].

ALK (OMIM 105590) is dominantly expressed in the neural system and the gene encoding it is located on the short arm of chromosome 2 (2p23.2) [4, 5]. It is a tyrosine kinase that was first identified as part of a fusion gene between *NPM* (nucleophosmin) and *ALK*, which is the result of the translocation t(2;5)(p23;q35) in anaplastic large cell lymphoma [6, 7]. The ALK kinase is constitutively activated by the gene amplification of *ALK* in three neuroblastoma cell lines and it has been shown that the suppression of activated ALK induces apoptosis through reduced phosphorylation of ShcC, mitogen-activated protein kinases and Akt [8]. The *ALK* locus has previously been reported to be amplified in single cases of primary neuroblastoma tumours with *MYCN* amplification [8-12]. In a recent study by Mosse et al., *ALK* was identified as a major familial neuroblastoma predisposition gene with activating germline mutations mapping to the TKD [13]. They also detected *ALK* amplifications in sporadic tumours mainly of metastatic stage and with poor outcome and *ALK* mutations in a subset of high-risk neuroblastoma [13]. We recently performed a CGH array study of 92 NB tumors [14] and detected a few cases of amplification of the *ALK* gene in chromosome region 2p that was distinct from the amplification of *MYCN* (also in 2p but distal of the *ALK* gene). These data together with the recent finding by Mosse et al. prompted us to perform this detailed study of *ALK* gene copy number, amplification, rearrangement and mutation as well as expression patterns of *ALK* and related genes in sporadic childhood neuroblastoma tumours of all clinical stages.

EXPERIMENTAL

Patient and control material

A panel of 90 primary NB tumours of all clinical stages according to the International Neuroblastoma Staging System, INSS, 11 stage 1, 12 stage 2, 13 stage 3, 46 stage 4, four stage 4S and four tumours of unknown stage from children without known familial history of neuroblastoma, was used for mutation analysis. In addition, normal tissue (blood samples) from some of the patients with identified mutations was used. Genomic DNA was extracted with a DNeasy blood and tissue kit (Qiagen, Hilden, Germany) according to the protocol provided by the supplier. RNA from 19 of the tumours used for mutation analysis was extracted from collected tumour material after homogenisation by TissueLyser (Qiagen), using the Totally RNA kit (Ambion, St. Austin, TX). Genomic DNA was removed with the DNA-free kit (Ambion) and the purity and integrity of the RNA samples were assayed with the ND-1000 spectrophotometer (NanoDrop, Wilmington, DE) and RNA 6000 Nano Bioanalyzer (Agilent, Palo Alto, CA) respectively. Seventy of the tumours used for mutation screening have also been analysed with SNP arrays, together with 38 additional tumours; this material has been described previously [14]. Informed consent was obtained from the patients' parents and the study was approved by the relevant ethics committees.

SNP microarray analysis

The DNA microarray experiments have been described previously [14]. Briefly, Affymetrix 250K arrays were used and primary data analysis was performed using GDAS software (Affymetrix, Inc., Santa Clara, CA), while further statistical studies were performed using CNAG (Copy Number Analyzer for Affymetrix GeneChip Mapping arrays) software, version 3.0 (GenomeLaboratory, Tokyo University, http://www.genome.umin.jp) [15, 16].

DNA amplification and sequencing

Primers were designed for exons and flanking intronic sequences of the TKD and surrounding exons of the *ALK* transcript (uc002rmy.1/ NM_004304 from UCSC Genome Browser March 2006; exons 20-26). Primers were designed using the Exonprimer feature of the UCSC genome browser (URL: http://genome.ucsc.edu/) and primers were ordered from Invitrogen (Invitrogen, Carlsbad, CA). Touch-down PCR was performed in 10µl reactions containing 1X Coral Load PCR Buffer (Qiagen), 0.2mM dNTP mix, 0.25U Hot Star TaqPlus DNA polymerase (Qiagen), 10µM of forward and reverse primer respectively and 25ng of tumour DNA. The PCR program was performed as follows: 95°C for five minutes before cycling 20 rounds of 95°C for 30 sec, 65°C for 30 sec (decreasing 0.5°C in every cycle) and 72°C for one minute – followed by 20 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for one minute and finally a 72°C extension step for seven minutes. The specificity of products was inspected by agarose gel electrophoresis before they were purified using Agencourt AMPure magnetic beads (Beckman Coulter, Beverly, MA) using the Biomek NX pipetting robot (Beckman Coulter) and eluted in dH_2O.

Sequence PCR was performed using the BigDye Terminator (BDT) v3.1 Cycle Sequence Kit (Applied Biosystems) in 10µl reactions containing 6µl 1:3 diluted PCR-template DNA, 1µl BDT, 1X BDT buffer and 1.6µM of forward or reverse PCR primer. Sequence PCR was run under the following conditions: 94°C for three minutes, followed by 50 cycles of 96°C for 30 sec, 50°C for 10 sec and 60°C for three minutes each. Sequencing products were purified using CleanSeq magnetic beads (Agencourt) using the Biomek NX and re-suspended in 10µl

of High Dye formamide (Applied Biosystems). The sequencing products were separated with gel electrophoresis on an 3730 DNA analyser (Applied Biosystems) and the output data were viewed and analysed using SeqScape v 2.5 (Applied Biosystems). All the fragments were analysed with both forward and reverse primer and all the findings were confirmed by sequencing a new PCR product.

Expression microarray analysis

Microarray expression analysis from 19 tumours including samples without *ALK* gene aberrations and samples with *ALK* gene rearrangement, amplification and mutation of the TKD was performed using the HU133A and HU133plus2 expression arrays from Affymetrix (www.affymetrix.com). Expression analysis of the five tumour cases, NB56, NB18, NB41, NB42 and NB30, was performed on HU133A chips as described previously (Wilzén et al., 2008, submitted), whereas the remaining 14 tumour cases were analysed by the HU133plus2 platform at Aros AB (www.arosab.com, unpublished data). The total RNA samples used for the HU133A chips were labelled according to standard procedures from Affymetrix, whereas the total RNA samples used for the HU133plus2 analysis were labelled using the NuGEN whole transcriptome amplification WT-Ovation™ FFPE System (www.nugenic.com).

The downstream genes assumed to be positively regulated by ALK were selected from Bohling et al. [17]. Gene profiles from two fusion gene experiments (TPM3-ALK and NPM-ALK) were compared, and ALK-specific downstream targets were identified. In the current study, we chose the eight most up-regulted genes from these expression profiles; *BCL10*, *CEBPB*, *INHBA*, *IL2RB*, *COL6A3*, *TNC*, *IL1R1*, and *FCGR3A/FCGR3B*, to represent the ALK-pathway activation. A Pearson correlation calculation was performed on our expression data from these eight genes, and they were found to correlate well. To add up the total activation of the ALK downstream pathway, the mean of the log2 expression value from the eight genes was calculated and then raised to the power of 2.

RESULTS

SNP array analysis

Four tumours harboured amplification of the *ALK* gene and one tumour showed a re-arrangement inside *ALK*, see Figure 1. In addition, *ALK* was amplified in the IMR-32 NB cell line. All cases with amplification also showed amplification of *MYCN*. A gain of chromosome 2p was detected in 20 cases; 18 of these included the *ALK* gene and five showed *MYCN* amplification, see Figure 2. Moreover, five NB cell lines showed a gain of chromosome 2p; two of them also had an amplification of *MYCN*. It should be noted that cell line SH-SY-5Y is derived from cell line SK-N-SH, and as expected they showed identical 2p-gain.

DNA sequencing

Five different missense mutations in a total of 10 tumours were detected in the TKD, see Table 1. In exon 20 (outside the TKD), at base 3182, a G>A substitution was detected in one NB tumour. This resulted in an amino acid change from arginine to a glutamine. At positions 3520 and 3522, the mutations T>A (one tumour) and C>A (three tumours), respectively, were detected, both changing the amino acid phenylalanine into isoleucine and leucine respectively (Figure 3). Two novel mutations, 3733T>A and 3735C>A (not previously published), were detected in exon 24, giving rise to an amino acid change from phenylalanine to isoleucine and leucine, in one tumour each. In four NB tumours, a mutation was detected at position 3824 (G>A), resulting in the transition from the amino acid arginine to glutamine. In addition, one silent change (3633C>A) was found in three tumours and one non-coding (IVS22+18 C>T) was detected in five tumours.

Expression analysis

Gene expression of ALK was analysed in 19 NB tumours by DNA microarrays, see Figure 4. The highest expression was detected in the three unfavourable tumours; NB29, NB42 and NB30 harbouring *ALK* mutation and amplifications, respectively. In addition, eight ALK-downstream positively regulated transcripts were analysed (Figure 4) and their expression levels correlated significantly ($p<0.01$) with one another according to Pearson's correlation test (data not shown). Results from six tumours, NB21, NB56, NB18, NB41, NB42, and NB30 indicate activation of both ALK and the ALK downstream pathway.

DISCUSSION

The *ALK* gene has been shown to be involved in several chromosomal translocations or inversions contributing to oncogenesis and providing a putative therapeutic target in several different tumour types, reviewed by Chiarle et al. [18]. The most common is the translocation t(2;5)(p23;q35) in anaplastic large cell lymphomas that gives rise to the oncogenic NPM-ALK fusion protein. Fusion proteins involving *ALK* and other partner proteins have also been identified in diffuse large B-cell lymphomas and inflammatory myofibroblastic tumours [19, 20].

From our study and recent data on neuroblastoma it is suggested that ALK is activated and may contribute to tumour development also through gene amplification and specific mutations targeting the TKD as indicated by elevated expression of ALK and downstream genes and constitutive kinase phosphorylation [13]. In a recent study, screening various molecular targeted inhibitors in a large panel of cell lines, it was found that a selective inhibitor of ALK, TAE684, potently suppresses the growth of a subset of neuroblastoma cell lines and the authors suggest that neuroblastoma tumours with *ALK* amplification or re-arrangements may be responsive to treatment with ALK kinase inhibition [21]. ALK is therefore an attractive target for novel therapeutic strategies in neuroblastoma and it is noteworthy that several ALK kinase inhibitors are in development for specific targeted cancer therapy including early clinical testing [22].

In a recent study using array CGH of 92 NB primary tumours, we identified rare cases with *ALK* amplification. In this study we more thoroughly investigated the frequency of *ALK* abnormalities in neuroblastoma using data from the array CGH, expression arrays and DNA sequencing. In our study, the first to investigate both mutations and copy number alterations in a large set of sporadic NB tumours of all clinical stages and different biological subsets, we detected *ALK* aberrations in a subset of advanced tumours from children with unfavourable outcome. *ALK* amplification and intragenic re-arrangements were detected in five tumours (Figure 1), giving a frequency of chromosomal abnormalities of 4.6% (5/108) with 3.7% being amplifications, close to the 3.3% amplification rate in sporadic cases recently published [13]. In addition, a previously not reported amplification of *ALK* was also detected in the IMR-32 NB cell line. Eighteen additional tumours harboured a gain of chromosome 2p that included *ALK* (16.7% compared to 22.8% as previously reported [13]), see Figure 2. Five of them also had an amplification of the *MYCN* gene. The gene target of the 2p gain in NB tumours is not known. Both *MYCN* and/or *ALK* are potent targets that could contribute to transformation when involving extra copies. In this context, it is interesting to note that 18 of 20 2p gain cases contain *ALK* in the gain region.

Mosse et al. sequenced the coding exons of *ALK* in NB and reported alterations in the TKD of *ALK* in familial neuroblastoma and in high-risk sporadic NB tumours [13]. In the present study, we also focused on the TKD and surrounding exons and sequenced them in 90 NB tumours. Mutations resulting in amino acid changes were found in 11 cases (10 located in the TKD), see Table 1. Three of the mutations in the TKD have been reported previously and they have a predicted high probability of resulting in oncogenic activation [13], while two have not been described previously. Interestingly, they target the same amino acid as one of the mutations previously reported, phenylalanine at amino acid position 1245. The frequency of amino acid substitutions in the TKD was 11.1% in our material of tumours from all clinical stages compared with 12.4% in high-risk tumours as investigated by Mosse et al. Counting DNA mutations inside the TKD and chromosomal abnormalities gives a frequency of 16% of

tumours where *ALK* is affected in the present study (2p gain cases not included). Interestingly, although investigating the full spectrum of NB tumours, *ALK* activating gene aberrations were only detected in advanced tumours either metastatic or with big primaries (large or unresectable stage 2 or 3 according to the INSS) including several with extensive tumour growth into the spinal canal (data not shown). Children with small localised tumours available for radical surgery never showed either mutations in the TKD, *ALK* amplifications or unbalanced 2p gain (p=0.002, Fisher's exact test). Furthermore, *ALK* aberrations correlated significantly with unfavourable outcome with inferior survival probability of 33%, 50% and 52% for children with TKD mutations, amplifications and 2p gain, respectively. However, all children dying from disease had additional unfavourable clinical and biological prognostic features, apart from *ALK* alterations. One patient, (harbouring the F1245I mutation; Table 1) with extensive regional residual tumour without known adverse prognostic factors, is a long term survivor despite absence of therapy for remaining viable tumour tissue. The clinical role of *ALK* mutations still seems unclear since both among familial cases [13] and in our material of sporadic tumours there are several long-term survivors indicating that *ALK* mutations is not an adverse factor *per se*.

The expression analysis agrees well with the data on gene amplification and DNA mutations. The three tumours with the highest expression of ALK had either *ALK* amplification (two cases), or a mutation (F1245L; one case; Figure 4). Moreover, high ALK expression appears to up-regulate ALK downstream targets, although the re-arrangement in tumour NB10 does not appear to affect the expression of ALK, and it only slightly affects downstream genes. Moreover, the F1245L mutation found in case NB29 does not appear to affect the ALK downstream targets selected in this study. This may be explained by the mutation-specific activation of selected downstream pathways. Also, the eight selected targets (see experimental section) probably do not cover all downstream effects of ALK. We should also bear in mind that we did not include analysis of ALK protein expression or phosphorylation status. Interestingly, there are four cases (NB21, NB56, NB18 and NB41) that both show high ALK expression and indicate strong downstream activation, but with no detectable *ALK* amplification or mutation. These cases will be the subject of further detailed mutation and re-arrangement studies of *ALK*.

In conclusion, we have analysed chromosomal re-arrangements of the *ALK* gene, performed a mutation screening of the *ALK* TKD and investigated the expression of ALK and related genes in a large number of sporadic NB primary tumours from all clinical and biological subsets. We present two novel mutations in the TKD of *ALK*. *ALK* mutations were found in 11% of NB tumours and gene amplification or re-arrangements were found in 5%. DNA mutations and gene amplifications generally showed a correlation with the expression level of ALK and downstream genes of ALK and unfavourable clinical features like extensive primary tumours or metastatic stage and poor prognosis. The data presented here and those previously published by Mosse et al. [13] provide clear evidence that ALK plays an important role in a significant fraction of advanced sporadic NB tumours as well as in the small number of cases with familial tumours. These studies also open new options for specific targeted therapy in children with neuroblastoma.

ACKNOWLEDGEMENTS

We would like to thank the Genomics Core Facility resource unit at the University of Gothenburg for access to the ABI 3730 Sequencer. This work has been supported by grants from the Swedish Cancer Society, the Children's Cancer Foundation, the King Gustav V Jubilee Clinic Cancer Research Foundation, the Assar Gabrielsson Foundation, the Wilhelm and Martina Lundgren Research Foundation, the Sahlgrenska University Hospital Foundation, Stockholm Cancer Society and Stockholm County Council. HC is the recipient of a fellowship from the Swedish Knowledge Foundation through the Industrial PhD Programme in Medical Bioinformatics at the Strategy and Development Office (SDO) at Karolinska Institutet, FA is the recipient of a post-doc position from the Swedish Medical Council and TM is the recipient of a senior cancer researcher position from the Swedish Cancer Society. We declare no competing interests.

REFERENCES

1 Brodeur, G. M., Sekhon, G. and Goldstein, M. N. (1977) Chromosomal aberrations in human neuroblastomas. Cancer. **40**, 2256-2263

2 Gilbert, F., Feder, M., Balaban, G., Brangman, D., Lurie, D. K., Podolsky, R., Rinaldt, V., Vinikoor, N. and Weisband, J. (1984) Human neuroblastomas and abnormalities of chromosomes 1 and 17. Cancer Res. **44**, 5444-5449

3 Schwab, M., Alitalo, K., Klempnauer, K. H., Varmus, H. E., Bishop, J. M., Gilbert, F., Brodeur, G., Goldstein, M. and Trent, J. (1983) Amplified DNA with limited homology to myc cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour. Nature. **305**, 245-248

4 Pulford, K., Lamant, L., Morris, S. W., Butler, L. H., Wood, K. M., Stroud, D., Delsol, G. and Mason, D. Y. (1997) Detection of anaplastic lymphoma kinase (ALK) and nucleolar protein nucleophosmin (NPM)-ALK proteins in normal and neoplastic cells with the monoclonal antibody ALK1. Blood. **89**, 1394-1404

5 Shiota, M., Fujimoto, J., Takenaga, M., Satoh, H., Ichinohasama, R., Abe, M., Nakano, M., Yamamoto, T. and Mori, S. (1994) Diagnosis of t(2;5)(p23;q35)-associated Ki-1 lymphoma with immunohistochemistry. Blood. **84**, 3648-3652

6 Morris, S. W., Kirstein, M. N., Valentine, M. B., Dittmer, K. G., Shapiro, D. N., Saltman, D. L. and Look, A. T. (1994) Fusion of a kinase gene, ALK, to a nucleolar protein gene, NPM, in non-Hodgkin's lymphoma. Science (New York, N.Y. **263**, 1281-1284

7 Shiota, M., Fujimoto, J., Semba, T., Satoh, H., Yamamoto, T. and Mori, S. (1994) Hyperphosphorylation of a novel 80 kDa protein-tyrosine kinase similar to Ltk in a human Ki-1 lymphoma cell line, AMS3. Oncogene. **9**, 1567-1574

8 Osajima-Hakomori, Y., Miyake, I., Ohira, M., Nakagawara, A., Nakagawa, A. and Sakai, R. (2005) Biological role of anaplastic lymphoma kinase in neuroblastoma. The American journal of pathology. **167**, 213-222

9 George, R. E., Attiyeh, E. F., Li, S., Moreau, L. A., Neuberg, D., Li, C., Fox, E. A., Meyerson, M., Diller, L., Fortina, P., Look, A. T. and Maris, J. M. (2007) Genome-wide analysis of neuroblastomas using high-density single nucleotide polymorphism arrays. PLoS ONE. **2**, e255

10 Stock, C., Bozsaky, E., Watzinger, F., Poetschger, U., Orel, L., Lion, T., Kowalska, A. and Ambros, P. F. (2008) Genes proximal and distal to MYCN are highly expressed in human neuroblastoma as visualized by comparative expressed sequence hybridization. The American journal of pathology. **172**, 203-214

11 Lastowska, M., Viprey, V., Santibanez-Koref, M., Wappler, I., Peters, H., Cullinane, C., Roberts, P., Hall, A. G., Tweddle, D. A., Pearson, A. D., Lewis, I., Burchill, S. A. and Jackson, M. S. (2007) Identification of candidate genes involved in neuroblastoma progression by combining genomic and expression microarrays with survival data. Oncogene. **26**, 7432-7444

12 Chen, Q. R., Bilke, S., Wei, J. S., Whiteford, C. C., Cenacchi, N., Krasnoselsky, A. L., Greer, B. T., Son, C. G., Westermann, F., Berthold, F., Schwab, M., Catchpoole, D. and Khan, J. (2004) cDNA array-CGH profiling identifies genomic alterations specific to stage and MYCN-amplification in neuroblastoma. BMC genomics. **5**, 70

13 Mosse, Y. P., Laudenslager, M., Longo, L., Cole, K. A., Wood, A., Attiyeh, E. F., Laquaglia, M. J., Sennett, R., Lynch, J. E., Perri, P., Laureys, G., Speleman, F., Kim, C., Hou, C., Hakonarson, H., Torkamani, A., Schork, N. J., Brodeur, G. M., Tonini, G. P., Rappaport, E., Devoto, M. and Maris, J. M. (2008) Identification of ALK as a major familial neuroblastoma predisposition gene. Nature. Aug 24. [Epub ahead of print] doi:10.1038/nature07261

14 Carén, H., Erichsen, J., Olsson, L., Enerbäck, C., Sjöberg, R. M., Abrahamsson, J., Kogner, P. and Martinsson, T. (2008) High-resolution array copy number analyses for detection of deletion, gain, amplification and copy-neutral LOH in primary neuroblastoma tumors; Four cases of homozygous deletions of the CDKN2A gene. BMC genomics. **9**, 353
15 Nannya, Y., Sanada, M., Nakazaki, K., Hosoya, N., Wang, L., Hangaishi, A., Kurokawa, M., Chiba, S., Bailey, D. K., Kennedy, G. C. and Ogawa, S. (2005) A robust algorithm for copy number detection using high-density oligonucleotide single nucleotide polymorphism genotyping arrays. Cancer Res. **65**, 6071-6079
16 Yamamoto, G., Nannya, Y., Kato, M., Sanada, M., Levine, R. L., Kawamata, N., Hangaishi, A., Kurokawa, M., Chiba, S., Gilliland, D. G., Koeffler, H. P. and Ogawa, S. (2007) Highly sensitive method for genomewide detection of allelic composition in nonpaired, primary tumor specimens by use of affymetrix single-nucleotide-polymorphism genotyping microarrays. Am J Hum Genet. **81**, 114-126
17 Bohling, S. D., Jenson, S. D., Crockett, D. K., Schumacher, J. A., Elenitoba-Johnson, K. S. and Lim, M. S. (2008) Analysis of gene expression profile of TPM3-ALK positive anaplastic large cell lymphoma reveals overlapping and unique patterns with that of NPM-ALK positive anaplastic large cell lymphoma. Leukemia research. **32**, 383-393
18 Chiarle, R., Voena, C., Ambrogio, C., Piva, R. and Inghirami, G. (2008) The anaplastic lymphoma kinase in the pathogenesis of cancer. Nature reviews. **8**, 11-23
19 Pulford, K., Lamant, L., Espinos, E., Jiang, Q., Xue, L., Turturro, F., Delsol, G. and Morris, S. W. (2004) The emerging normal and disease-related roles of anaplastic lymphoma kinase. Cell Mol Life Sci. **61**, 2939-2953
20 Gascoyne, R. D., Lamant, L., Martin-Subero, J. I., Lestou, V. S., Harris, N. L., Muller-Hermelink, H. K., Seymour, J. F., Campbell, L. J., Horsman, D. E., Auvigne, I., Espinos, E., Siebert, R. and Delsol, G. (2003) ALK-positive diffuse large B-cell lymphoma is associated with Clathrin-ALK rearrangements: report of 6 cases. Blood. **102**, 2568-2573
21 McDermott, U., Iafrate, A. J., Gray, N. S., Shioda, T., Classon, M., Maheswaran, S., Zhou, W., Choi, H. G., Smith, S. L., Dowell, L., Ulkus, L. E., Kuhlmann, G., Greninger, P., Christensen, J. G., Haber, D. A. and Settleman, J. (2008) Genomic alterations of anaplastic lymphoma kinase may sensitize tumors to anaplastic lymphoma kinase inhibitors. Cancer Res. **68**, 3389-3395
22 Li, R. and Morris, S. W. (2008) Development of anaplastic lymphoma kinase (ALK) small-molecule inhibitors for cancer therapy. Medicinal research reviews. **28**, 372-412

LEGENDS TO FIGURES

Figure 1. Amplification of the *ALK* gene in NB tumours and cell lines.

Figure 2. Gain of chromosome 2p in 20 NB tumours and five NB cell lines. Note that cell line SH-SY-5Y is derived from cell line SK-N-SH.

Figure 3. Missense mutations in the TKD of the *ALK* gene in neuroblastoma primary tumours. For each mutation, the upper panel shows chromatogram of normal tissue from a patient with the mutation (* when available), the middle panel shows the mutation in primary tumours and the lower a reference sequence. The tumour case represented in the lower panel for each mutation is underlined.

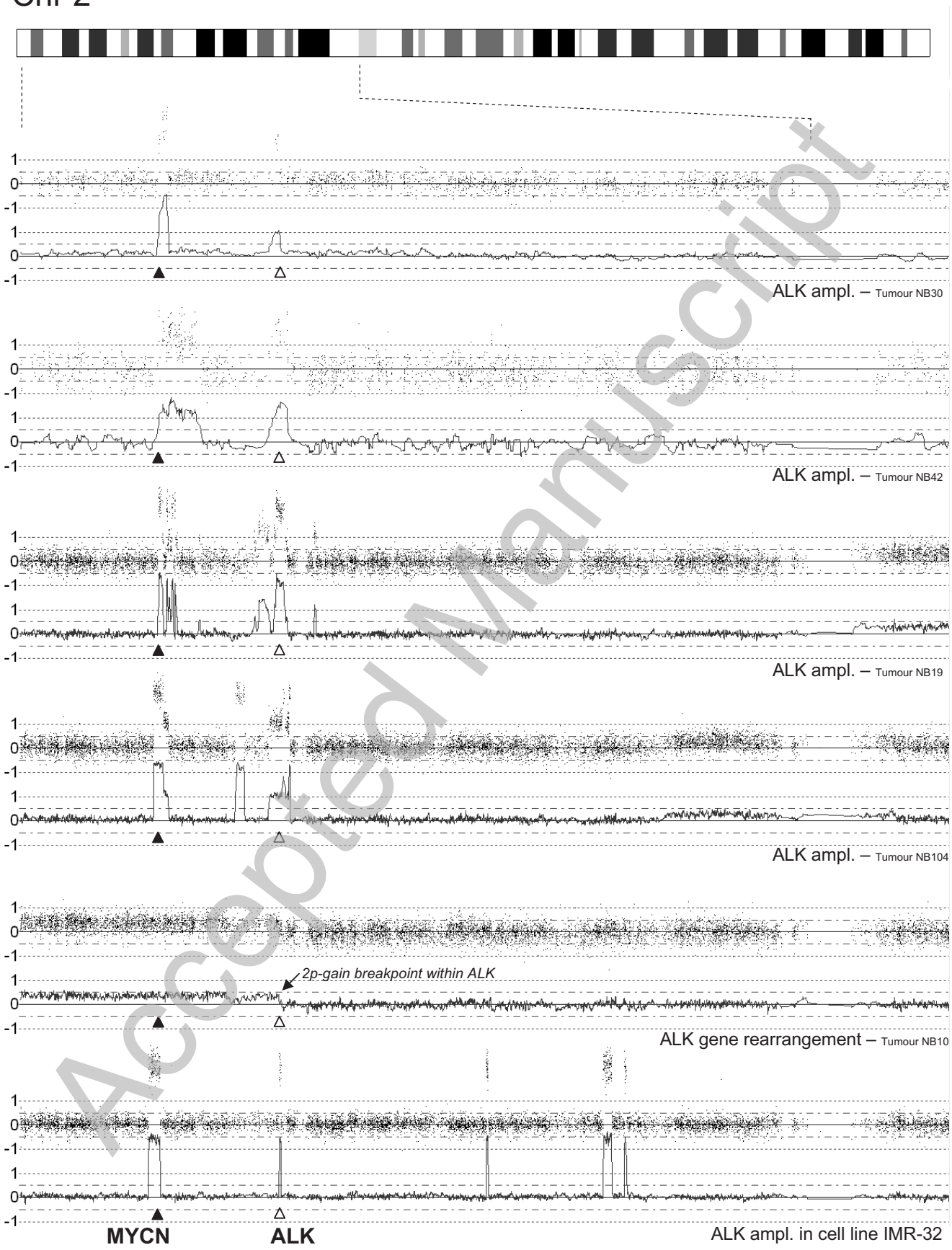
Figure 4. Relative ALK expression from 19 NB tumours. The expression values are generated from Affymetrix HU133A or HU133plus2 expression arrays (Wilzén et al., 2008, submitted and unpublished data). Blue bars represent the relative ALK expression. Lines represent the mean expression from eight ALK-downstream positively regulated transcripts (see text for details). Light bars (white dotted) represent favourable tumour cases, while dark bars (grey squared) represent unfavourable tumours. Cases showing genomic amplification (NB30, NB42) or a re-arrangement (NB10) of ALK and the tumour case showing a mutation in the TKD of *ALK* (NB29) are marked.

Table 1. DNA mutations and chromosomal abberations in the *ALK* gene.

	Gene position	Base change	Predicted protein change	Patient	NB stage	Outcome	Survival after diagnosis	Additional Chr 2 features	Normal tissue from the patient
Mutation	Exon 23	3520T>A	F1174I	NB92	2	AWD	4+	neg	n.a.
		3522C>A	F1174L	NB27	4	DOD	8	MNA	n.a.
		3522C>A	F1174L	NB28	4	DOD	10	MNA	n.a.
		3522C>A	F1174L	NB36	4	DOD	29	MNA	C/C
	Exon 24	3733T>A	F1245I	NB61	2	AWD	66+	neg	n.a.
		3735C>A	F1245L	NB29	3	DOD	21	WCG	C/C
	Exon 25	3824G>A	R1275Q	NB64	4	AWD	2+	MNA	n.a.
		3824G>A	R1275Q	NB70	4	NED	30+	2p-gain	G/G
		3824G>A	R1275Q	NB90	4	AWD	4+	MNA	n.a.
		3824G>A	R1275Q	NB91	4	NED	4+	MNA	n.a.
Amplification				NB30	4	DOD	12	MNA	
				NB42	3	NED	127+	MNA	
				NB19	4	DOD	3	MNA	
				NB104	4	NED	14+	MNA	
Structural abberation				NB10	4	NED	190+	2p-gain	

AWD, alive with disease; DOD, dead of disease; NED, no evidence of disease; Chr2, chromosome 2; neg, negative; MNA, MYCN amplification; WCG, whole chromosome gain; n.a., not available

Biochemical Journal Immediate Publication. Published on 07 Oct 2008 as manuscript BJ20081834



Biochemical Journal Immediate Publication. Published on 07 Oct 2008 as manuscript BJ20081834