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mRNA Expression Level of ALK in Neuroblastoma Is Associated with Histological Subtype, ALK Mutations and ALK Immunohistochemical Protein Expression

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Abstract: *ALK* is related to poor survival in neuroblastoma patients. We investigated the prognostic relevance of *ALK* mRNA expression and the relationship with ALK immunohistochemical expression, histological subtype and *ALK* aberrations. Whole transcriptome sequencing data were available from 54 patients. Overall survival (OS) and event-free survival (EFS) were estimated with Kaplan–Meier's methodology. ALK protein expression was analyzed by immunohistochemistry. *ALK* aberrations were detected using whole exome sequencing, single nucleotide polymorphism array, next generation sequencing and/or fluorescence in situ hybridization. OS was 74.8% and EFS was 60%. *ALK* mRNA expression was not associated with OS (HR 1.127, 95% CI (0.812–1.854), $p = 0.331$) and adjusted EFS (HR 1.134, 95% CI (0.783–1.644), $p = 0.505$), but was associated with histological subtype (OR 1.914, 95% CI (1.083–3.382), $p = 0.025$) and ALK protein expression (negative versus weak: OR 2.829, 95% CI (1.290–6.204), $p = 0.009$) (negative versus moderate/strong: OR 2.934, 95% CI (0.889–9.679), $p = 0.077$). *ALK* mutated tumors had significantly higher *ALK* mRNA expression than non-mutated tumors ($p < 0.001$). *MYCN*-amplified neuroblastomas have higher *MYCN* mRNA expression ($p \leq 0.001$), but not *ALK* mRNA expression ($p = 0.553$). *ALK* mRNA expression is higher in *ALK* mutated neuroblastomas and is associated with poorer differentiation degree and higher protein expression. *ALK* mRNA expression is not significantly associated with OS and EFS.

Keywords: neuroblastoma; ALK; whole transcriptome sequencing; mRNA sequencing; protein expression

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1. Introduction

Neuroblastoma spectrum tumors, in this article referred to as neuroblastomas, are the most common solid, extra-cranial, pediatric tumors [1]. They derive from the sympathetic nervous system and are responsible for 15% of cancer-related deaths in children [1,2]. However, the mortality rate for neuroblastomas is remarkably variable. Some tumors spontaneously regress, while others are extremely aggressive [3]. Once a relapse occurs, there are limited treatment options and survival rates are around 20% [4]. Therefore, it is of paramount importance to predict the aggressiveness of a neuroblastoma at diagnosis and determine a treatment strategy based on this prediction. Over the years it has become clear that molecular mechanisms play an essential role in defining subgroups of neuroblastomas. The International Neuroblastoma Risk Group (INRG) Classification System includes both

MYCN amplification and 11q aberration [5]. More recent studies have identified the ALK gene to be related to tumorigenesis, progression and treatment of neuroblastomas as well [6–13].

Anaplastic Lymphoma Kinase (ALK), a receptor tyrosine kinase, is an enzyme encoded by the *ALK* gene. It has pivotal connections to major signaling pathways, such as RAS/MAPK, PI3K/AKT and JAK/STAT [14–16]. In neuroblastoma cells it is most likely involved in neural differentiation [17,18]. Despite the extensive literature on *ALK* in neuroblastoma, the correlation between *ALK* gene expression and ALK immunohistochemical protein expression specifically, has not been established yet. While generally mRNA is transcribed to protein, in many instances there is no linear relationship between mRNA and protein expression, due to differential translation of mRNA to protein and due to differential splicing of mRNA [19–21]. Furthermore, analyzing immunohistochemical stains is not an exact method. Even experienced pathologists show high interobserver variation with regard to IHC scoring [22–24]. Whole transcriptome sequencing, which we routinely use in the diagnostic setting in our pediatric oncology center, could potentially serve as a more accurate method to determine *ALK* expression.

Furthermore, *ALK* mRNA expression levels are higher in neuroblastomas harboring a mutant or amplified *ALK* compared to wild-type *ALK* [3,25]. *ALK* activating point-mutations are common in both hereditary and non-hereditary neuroblastomas [6,8,11]. These mutations are located in the ALK tyrosine kinase domain and lead to ALK activation independent of ligand binding. Neuroblastoma cells carrying these mutations form tumors and continue to proliferate [26]. Mutants in *ALK* activate ERK5, through the PI3K/AKT/mTOR and MAPK pathways, which stimulate *MYCN* transcription and de novo MYCN protein expression. This results in proliferation and activation of apoptotic pathways [27–29]. Furthermore, activation of the RAS/MAPK pathway, results in increased ETV5 levels, resulting in proliferation of neuroblastoma cells [30]. Also, *ALK* activation (e.g., *ALK* activation point-mutations or *ALK* amplification) can inhibit *DLG2* transcription via ERK1/2. DLG2 inhibits tumor growth and drives differentiation in neuroblastomas. This theory is supported by research in mouse models, which shows that ALK activation blocks the differentiation of neuroblastoma cells. This leads to undifferentiated or poorly differentiated neuronal cells, rather than differentiated neuroblastomas [31]. Another possible mechanism of ALK activation are genomic rearrangements of *ALK*. Several studies have described overexpression of a truncated form of the ALK protein, in which part of the extracellular domain is lacking. This is due to amplification of an abnormal *ALK* gene depicting a deletion of exon 2–3 [32] or 4–11 [33,34].

The association between histological subclassification of neuroblastomas and survival has long been established and is incorporated in the International Neuroblastoma Risk Group (INRG) Stratification Classification System [5]. If *ALK* mRNA expression levels are indeed linked to differentiation degree of neuroblastomas, it would suggest that *ALK* mRNA expression is also linked to prognosis, which has been established already for neuroblastic tumors harboring an *ALK* genetic aberration [6,8,12,13]. Several studies have investigated the prognostic relevance of *ALK* mRNA expression levels and have shown a correlation with poor survival [3,35–37].

ALK is located at chromosome region 2p23, which is in close proximity to *MYCN* at region 2p24.1. A gain of chromosome arm 2p is associated with *ALK* amplification and *MYCN* amplification and is correlated with poor prognosis [38]. There are studies that suggest ALK activation only correlates with prognosis, because it potentiates acceleration of MYCN activity through the PI3K/AKT pathway [29,39].

Because of its oncogenic role and the availability of specific targeting drugs, *ALK* is subject to targeted therapy of neuroblastic tumors. Recent studies have investigated the efficacy of ALK tyrosine kinase inhibitors (TKIs) in neuroblastomas harboring *ALK* aberrations. Patient treated with lorlatinib showed a response rate of 30% <18 years and 67% >18 years, which is superior compared to first and second generation TKIs (crizotinib: 15% and ceritinib: 20%) [40–42]. Currently, neuroblastomas with high *ALK* mRNA expression

or immunohistochemical protein expression, but without *ALK* aberrations, are not suitable for ALK-inhibitor treatment [36].

In this retrospective study, we examined the relationship between mRNA expression levels of *ALK* and event-free survival (EFS) and overall survival (OS) in children with a neuroblastoma. Furthermore, we investigated whether *ALK* mRNA expression levels correlate with immunohistochemical expression of ALK. In addition, we investigated whether *ALK* mRNA expression levels are higher in less differentiated neuroblastomas, when the tumor is *ALK* amplified or when it carries an *ALK* mutation.

2. Materials and Methods

2.1. Study Population and Clinicopathological Data

Pathological and molecular data from patients diagnosed with a neuroblastoma in the Princess Máxima Center (PMC) between November 2014 and December 2021 were retrospectively collected. The requirement to obtain informed consent was waived by the Medical Ethical Committee, based on the Dutch Medical Research with Human Subjects Law. Data collection included core needle biopsies, excisional biopsies, incisional biopsies and resections. Tumors were classified according to the International Neuroblastoma Pathology Classification (INPC). Exclusion criteria were no whole transcriptome sequencing was performed on the sample, samples were not taken for diagnostic purposes, samples were obtained elsewhere, the clinical question did not concern neoplasia (e.g., clinical question concerned a possible graft versus host reaction), non-diagnostic samples, bone marrow biopsies, fine needle aspirates, autopsy samples, samples that showed >90% necrosis or samples without tumor cells. This yielded a total of 54 diagnostic samples. Clinicopathological data were obtained from clinical medical records, pathology reports and radiology reports. This included age at diagnosis, sex, OS, EFS, tumor location, type of material, clinical question and tumor classification using the INPC. OS was based on last contact with patients or their parents. EFS was based on the latest imaging performed (ultrasound, CT-scan or MRI). An event was defined as progression or relapse of the tumor, secondary malignancy or death. Tumor classification was determined by experienced pathologists working in the PMC. The tumors were categorized into two groups: group A = undifferentiated, poorly differentiated neuroblastoma and neuroblastoma not further specified and group B = differentiated neuroblastoma, ganglioneuroblastoma and ganglioneuroma.

2.2. Immunohistochemistry

Immunohistochemistry (IHC) was performed on formalin-fixed, paraffin-embedded (FFPE) tumor slides. The immunostaining is performed on an automated BOND-RX system (including the deparaffinization (leica Micorsystems). Antigen retrieval was performed by boiling the sections in TRIS/EDTA (BOND Epitope Retrieval Solution 2, pH9; Leica biosystems, Leica Microsystems, Wetzlar, Germany; Leica Biosystems, Amsterdam, The Netherlands) for 20 min. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in demineralized water for 5 min and the sections were incubated for 20 min at RT with the primary antibody ALK, dilution 1:100. The following antibody was used: 5A4 (Mouse) (Leica Biosystems, Richmond, IL, USA). The sections were then incubated for 8 min with a post primary rabbit anti-mouse linker followed by incubation for 8 min with anti-rabbit horseradish-peroxidase labeled polymer. After incubation for 10 min with diaminobenzidine, the slides were counterstained for 5 min with hematoxylin (BOND Polymer Refine Detection Kit; Leica Biosystems), dehydrated, cleared and mounted. On each slide, a positive and negative control tissue was included. ALK-translocated (2:5) anaplastic large-cell lymphoma tissue was used as a positive control. The negative control consisted of a normal lymph node sample.

The intensity and extent of ALK immunohistochemical protein expression were primarily scored by one of the authors (R.S.B.) and independently checked by an experienced pathologist (R.R.d.K.). Both cytoplasmic and membranous staining were taken into account.

Intensity was scored as follow: 0 (no staining), 1 (weak staining), 2 (moderate staining) and 3 (strong staining). The extent was scored in percentages. H-scores were calculated as the product of staining intensity and percentage, yielding a minimum score of 0 and a maximum score of 300. The H-scores were then split into four categories: negative protein expression: 0 to 75, weak protein expression: 75 to 150, moderate protein expression: 150 to 225 and strong protein expression 225 to 300 (Figure 1). Interventional studies involving animals or humans, and other studies that require ethical approval, must list the authority that provided approval and the corresponding ethical approval code.

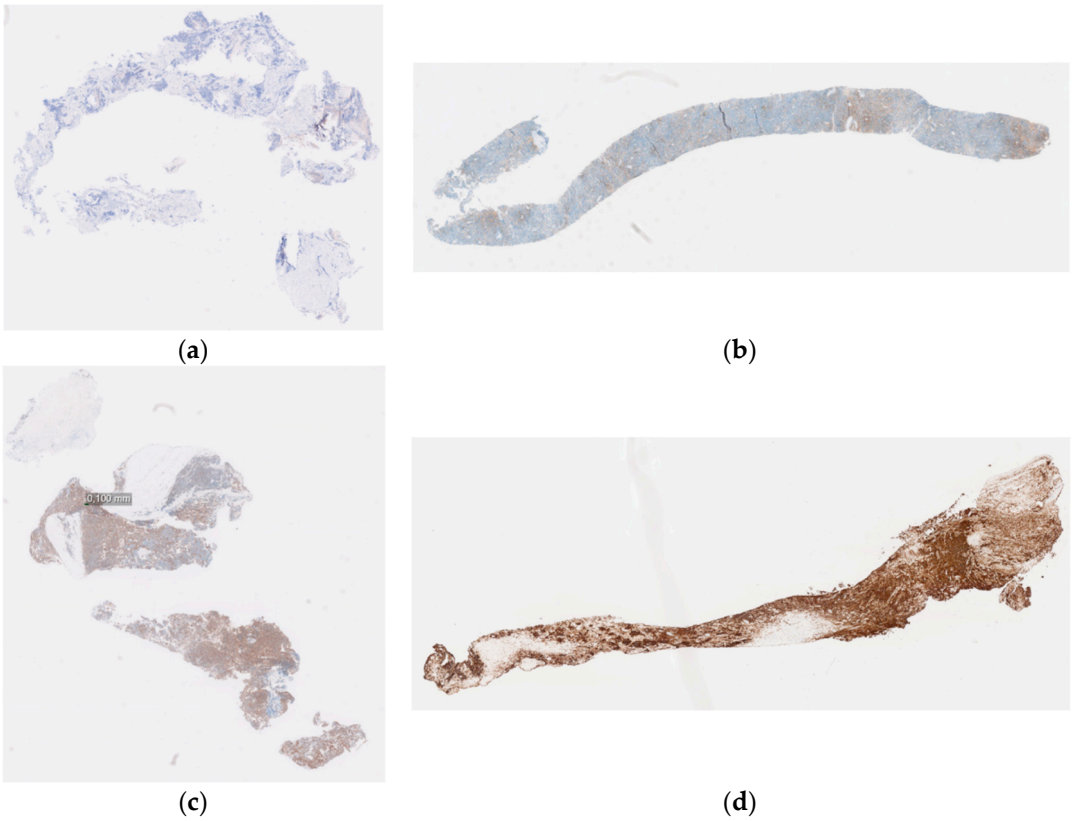


Figure 1. ALK immunohistochemical protein expression classified in H-scores: (**a**) negative protein expression: 0 to 75, (**b**) weak protein expression: 75 to 150, (**c**) moderate protein expression: 150 to 225 and (**d**) strong protein expression: 225 to 300.

2.3. Fluorescence In Situ Hybridization

Fluorescence In Situ Hybridization (FISH) was used to determine the copy number status ALK (in some cases) and MYCN for primary tumor biopsies at diagnosis from April 2015 to February 2017. The following probes were used: ALK break-apart probe LPS 019 (2p23.2p23.1, CytoCell, Cambridge, UK) and the MYCN amplification probe LPS 0009 (2p24.3, CytoCell, Cambridge, UK). FISH was performed as reported previously [43]. The slides were examined by a clinical scientist in molecular pathology using a fluorescence microscope as part of routine diagnostics.

MYCN amplification was defined as a MYCN/centromere ratio >4 (Figure 2), a gain as a ratio between 1.5–4 and a normal copy number status was defined as a ratio <1.5. ALK amplification was defined as >9 signal pairs, a gain as 3–8 signal pairs and the ALK copy number status was normal when there were 1–2 signal pairs.

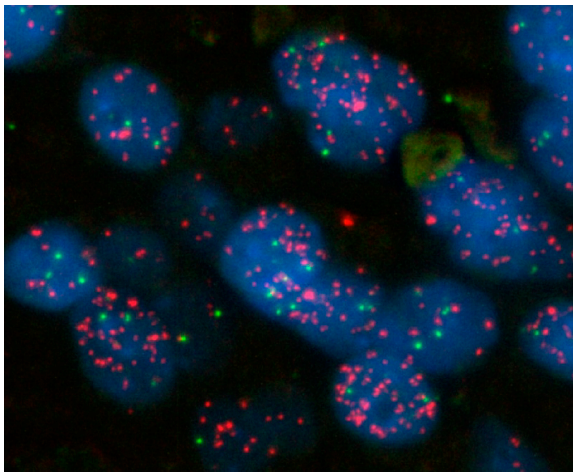


Figure 2. MYCN amplification detected by fluorescence in situ hybridization.

2.4. Single Nucleotide Polymorphism Array

From July 2017 till the end of recruitment, the copy number status of all neuroblastomas was determined using the Infinium CytoSNP-850 K BeadChip (Illumina, San Diego, CA, USA) according to standard procedures. Visualisation of SNP array results and data analysis was performed using NxClinical (BioDiscovery, El Segundo, CA, USA). Chromosome arms 13p, 14p, 15p, 21p and 22p do not have any SNPs, so these arms could not be analyzed. Chromosome X and Y were not checked for CNVs. The other chromosomes were analyzed by one of the authors (R.S.B.) and compared with a control group. This was independently checked by a molecular biologist (W.W.J.d.L.). When more than two copies of (a part of) a chromosome, or chromosome arm, were present, it was considered a gain or partial gain. Less than two copies were identified as a loss or partial loss.

Specific attention was paid to chromosome arm 2p, which contains the MYCN and ALK gene, to check for amplifications of one or both genes (Figure 3). Identifying these amplifications was completed by a molecular biologist (W.W.J.d.L.). It was considered an amplification when more than four copies of the ALK and/or MYCN gene were present.

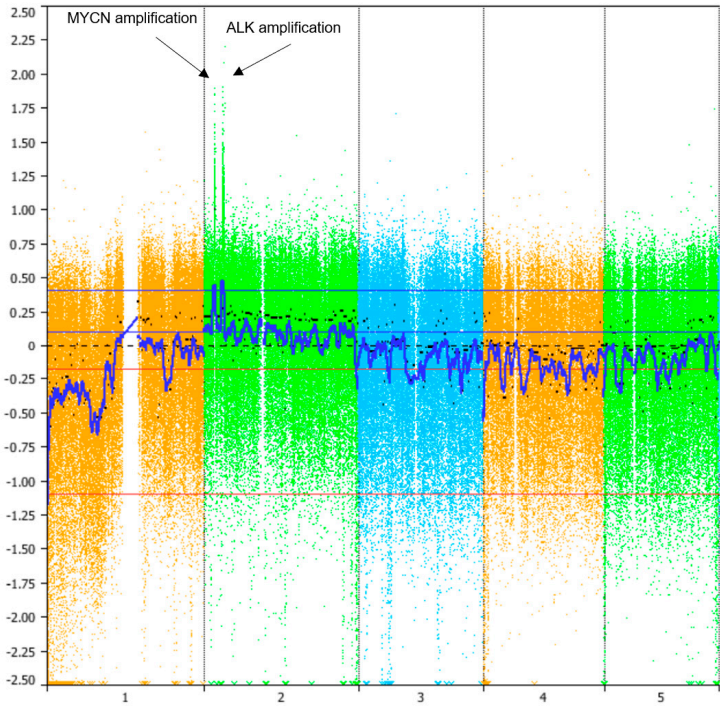


Figure 3. ALK and MYCN amplification detected by Single Nucleotide Polymorphism array.

2.5. Next Generation Sequencing

ALK mutational status was determined by Next Generation Sequencing (NGS). NGS was performed as previously described using the Cancer Hotspot Panel (Thermo Fisher Scientific, Waltham, MA, USA) in combination with IonTorrent sequencing (Thermo Fisher) [44]. Mutations where classified using terminology analogous to the American College of Medical Genetics and Genomics system for interpretation of sequence variants: benign, likely benign, variant of uncertain significance, likely pathogenic and pathogenic [45]. Both pathogenic and likely pathogenic ALK mutations were included in the database.

2.6. Whole Exome Sequencing

Total DNA was isolated using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Hilden, Germany) according to standard protocol on the QiaCube (Qiagen). DNA-seq libraries were generated with 150 ng DNA using the KAPA HyperPrep Kit in combination with the HyperExome capture kit (Roche, Basel, Switzerland) and subsequently sequenced on an NovaSeq 6000 system (2 × 150 bp) (Illumina, San Diego, CA, USA). The DNA sequencing data of the tumor and the normal DNA (extracted from blood) were processed as per the GATK 4.0 best practices workflow for variant calling, using a wdl- and cromwell-based workflow [46]. This included performing quality control with Fastqc (version 0.11.5) to calculate the number of sequencing reads and the insert size Picard (version 2.20.1) for DNA metrics output and MarkDuplicates [47]. A molecular biologist of the PMC (L.A.K.) analyzed all DNA sequencing data for possible mutations.

Single nucleotide variants (SNVs) were identified using paired tumor-normal samples by Mutect2 from GATK 4.1 [48] and pathogenicity was predicted by variant effect predictor (VEP) (version 92) [49]. Somatic copy number alterations (CNAs) were identified with the GATK4 pipeline using an in-house generated panel of normals (PON) from normal samples prepared and sequenced under the same conditions and used this for normalization. The allelic imbalance ratios were calculated using 1000 genomes, autosomal SNP sites with a minor allele frequency (MAF) > 0.1. One of the authors (R.S.B.) analyzed the data for CNV in all chromosomes, with the exception of the two sex chromosomes (X and Y). Specific attention was paid to chromosome arm 2p, which contains the MYCN and ALK gene, to check for amplifications of one or both genes (Figure 4). The results of the analyses were independently checked by an experienced molecular biologist (L.A.K.).

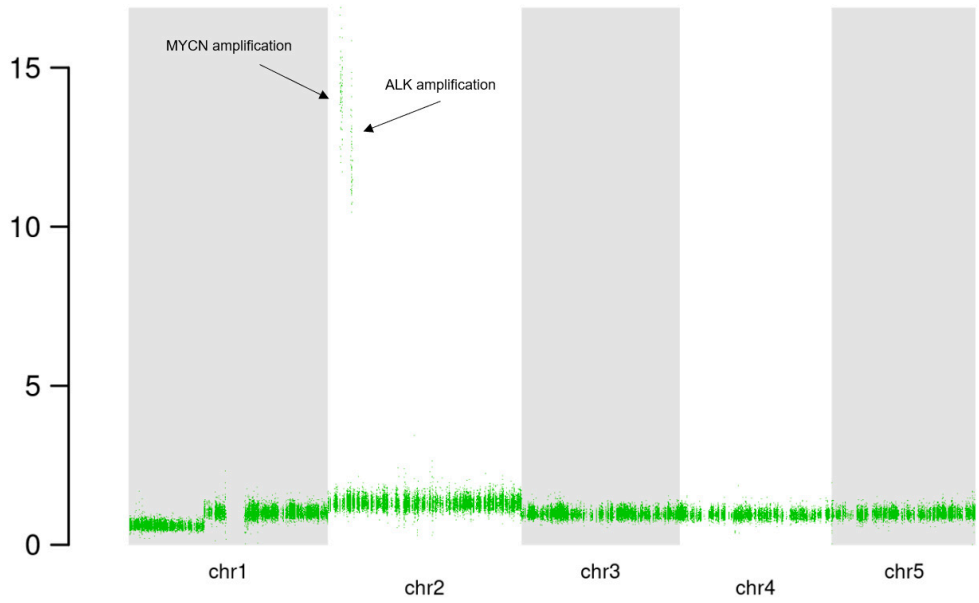


Figure 4. ALK and MYCN amplification detected by Whole Exome Sequencing.

2.7. Whole Transcriptome Sequencing

Whole transcriptome sequencing, or RNA sequencing, was performed as previously described [50]. In brief, total RNA was isolated using the AllPrep DNA/RNA/Protein Mini Kit (QIAGEN) according to standard protocol on the QiaCube (Qiagen). RNA-seq libraries were generated with 300 ng RNA using the KAPA RNA HyperPrep Kit with RiboErase (Roche) and subsequently sequenced on an NovaSeq 6000 system (2 × 150 bp) (Illumina). The RNA-sequencing data were processed as per the GATK 4.0 best practices workflow for variant calling, using a wdl- and cromwell-based workflow [46]. This included performing quality control with Fastqc (version 0.11.5) to calculate the number of sequencing reads and the insert size Picard (version 2.20.1) for RNA metrics output and MarkDuplicates. The raw sequencing reads were aligned using STAR (version 2.7.0f) to GRCh38 and gencode version 29 [50]. Finally, expression counts were determined at the gene level using Subread Counts [51].

For this study, the counts per million reads mapped (CPM) of the ALK gene and the MCYN gene were determined using RNA sequencing. Furthermore, all genomic rearrangements were reported. All data were analyzed by a molecular biologist of the PMC (L.A.K.).

2.8. Statistical Analysis

A combination of molecular techniques was often used to detect ALK aberrations. In case of inconsistency, the results of the WES were used for statistical analyses. When WES was not performed, ALK mutation status was based on NGS and ALK amplification status was based on SNP-array. FISH results were only used when this molecular technique was exclusively used.

Kaplan–Meier's methodology was employed to estimate OS and EFS since diagnosis. Reverse Kaplan–Meier estimate was used to determine the median follow up [52]. A Cox proportional Hazard regression model was used to estimate the effect of mRNA expression of ALK on OS and EFS. Hazard Ratio (HR) along with 95% confidence intervals (CI) were reported. MYCN amplification was incorporated in the Cox model for EFS. A log transformation of mRNA expression level of ALK was performed and this variable was incorporated in the Cox models, and all furthers statistical analyses. One outlier regarding mRNA expression level was excluded from the analyses.

A univariate logistic regression model was estimated to study the relationship between ALK gene expression and the diagnosis category. To test the relationship between mRNA expression and immunohistochemical protein expression of ALK, a multinomial logistic regression was performed. Odds ratio (OR) along with 95% CI were reported. For the relationship between mRNA expression of ALK and ALK mutations, an independent sample t-test was performed for which the p-value was provided. The same test method was applied for the relationship between ALK mRNA expression and gain of chromosome 2 or chromosome arm 2p and also for the relationship of ALK/MYCN mRNA expression levels with MYCN status.

SPSS for Windows, version 26.0.0.1. (IBM, Armonk, New York, NY, USA) was used to perform statistical analyses. A p-value < 0.05 was considered statistically significant.

3. Results

A total of 54 diagnostic samples were included in this study (Figure 5). The mean age at diagnosis was 2 years (range: 0–16 years). Poorly differentiated neuroblastoma was the most common histological subtype (Table 1). IHC using an ALK antibody was performed in 49 cases. Additional molecular analyses (besides whole transcriptome sequencing) were performed in 53 cases; FISH was successfully performed in 35 cases, NGS in 28 cases, SNP-array in 27 cases and WES in 36 cases. A total of 7 *ALK* mutations, one *ALK* amplification and 12 *MYCN* amplifications were detected. Further clinical characteristics are shown in Table 1.

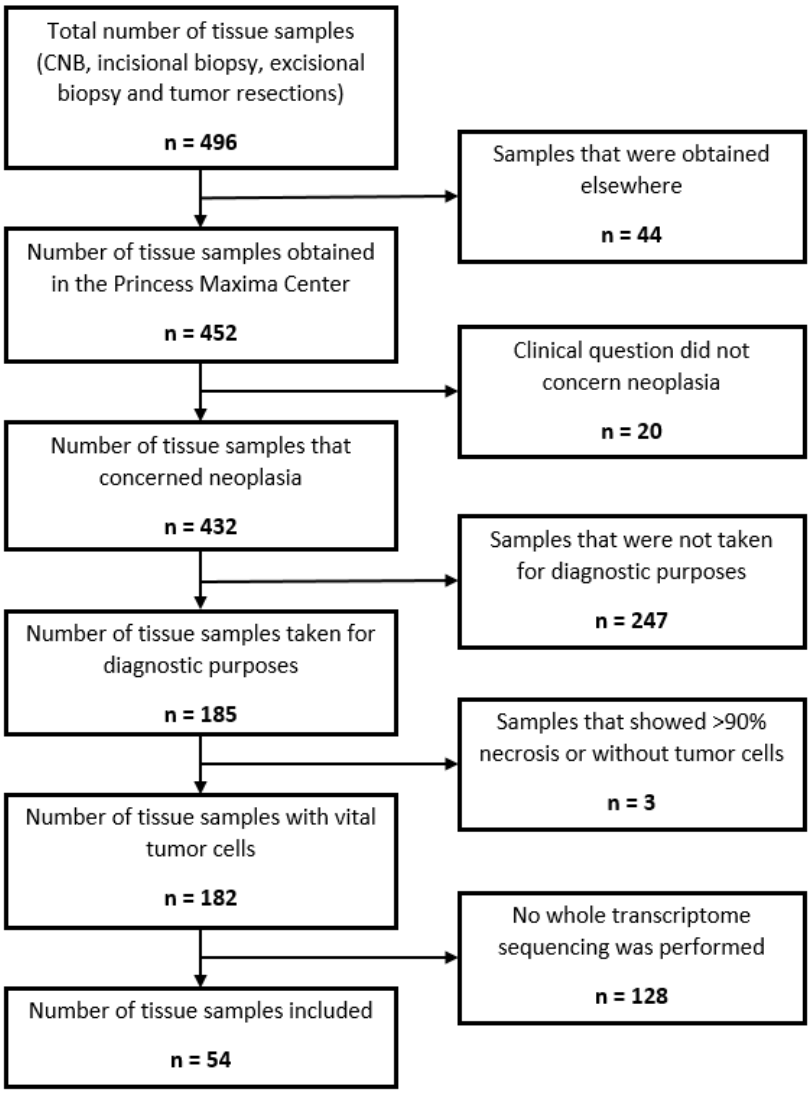


Figure 5. Flowchart inclusion tissue samples. This flowchart shows the exclusions of eligible tissue samples based on the exclusion criteria. Abbreviations: CNB, Core needle biopsy.

Table 1. Patient characteristics.

	N (%)
Gender	
Female	29 (54)
Male	25 (46)
Age group	
Infant (<1 year)	16 (30)
Child (1–18 years)	38 (70)
Overall survival	
Yes	41 (76)
No	13 (14)
Event free survival	
Yes	20 (63)
No	34 (37)
Primary tumor location	
Adrenal gland	29 (54)
Paravertebral ganglia	17 (32)
Other/unknown	8 (15)

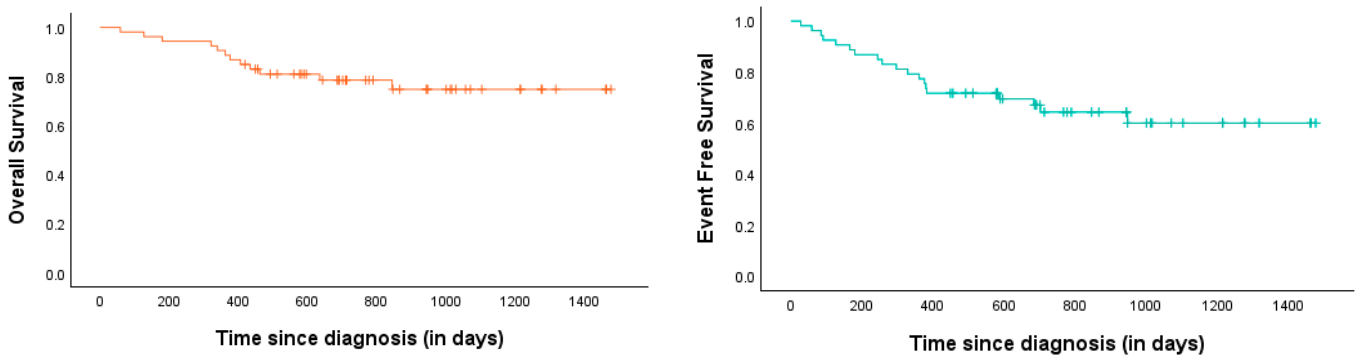
Table 1. *Cont.*

	N (%)
Classification (INPC)	
Neuroblastoma (not further specified)	2 (4)
Undifferentiated neuroblastoma	5 (9)
Poorly differentiated neuroblastoma	39 (72)
Differentiating neuroblastoma	4 (7)
Ganglioneuroblastoma	2 (4)
Ganglioneuroma	2 (4)
ALK protein expression	
Negative	33 (61)
Weak	11 (20)
Moderate	2 (4)
Strong	3 (6)
Molecular aberration	
Gain of 2(*p*)	19 (35)
ALK mutation	7 (13)
ALK amplification	1 (2)
MYCN amplification	12 (22)

Abbreviations: ALK, anaplastic lymphoma kinase; CI, confidence interval; EFS, event free survival; and OS, overall survival.

3.1. Prognostic Value of ALK mRNA Expression

In the whole cohort, the OS was 75% (SE 6.6) and the EFS was 63% (SE 7.6) since diagnosis (Figure 6). The median follow-up time for OS was 846 days (95% CI (630.764–1061.236)). Five patients died because of resistant disease, five patients experienced a relapse and treatment-related toxicity was the cause of death for three patients. Median follow up time for EFS was 866 days (95% CI (648.195–1084.805)). The most frequent first event was a relapse (n = 9), followed by progression (n = 7) and death (n = 4).



(**a**) (**b**)

Figure 6. Survival after diagnosis with a neuroblastic tumor estimated with Kaplan–Meier. (**a**) The overall survival (in days) and (**b**) event-free survival (in days).

A Cox proportional hazard model was estimated with ALK mRNA expression as risk factor for OS and both ALK mRNA expression and MYCN amplification for EFS. ALK mRNA expression does not affect the OS of children with a neuroblastoma (HR 1.123, 95% CI (0.812–1.854), p = 0.331). ALK mRNA expression is not related to EFS when adjusted for MYCN amplification (HR 1.134, 95% CI (0.783–1.644), p = 0.505) or unadjusted (HR 1.241, 95% CI (0.890–1.730), p = 0.204). MYCN amplification was not significantly associated to EFS (HR 2.301, 95% CI (0.894–5.926), p = 0.084). Results of the Cox proportional hazard model are shown in Table 2.

Table 2. Prognostic value of ALK mRNA expression and MYCN amplification.

	OS		EFS	
	HR	**95% CI (*p*-Value)**	**HR**	**95% CI (*p*-Value)**
ALK mRNA expression *Unadjusted*	1.127	0.812–1.854 (0.331)	1.241	0.890–1.730 (0.204)
ALK mRNA expression *Adjusted for MYCN amplification*			1.134	0.783–1.644 (0.505)
MYCN amplification *Adjusted for ALK mRNA expression*			2.301	0.894–5.926 (0.084)

ALK, anaplastic lymphoma kinase; CI, confidence interval; EFS, event free survival; and OS, overall survival.

3.2. mRNA Expression of ALK and Histological Sub Classification

The median CPM of ALK for group A (undifferentiated, poorly differentiated neuroblastoma and neuroblastoma not further specified) was 159 (n = 45) and 41 (n = 8) for group B (differentiated neuroblastoma, ganglioneuroblastoma and ganglioneuroma). Univariate logistic regression showed an association between ALK mRNA expression levels and differentiation degree of the tumor (OR 1.914, 95% CI (1.083–3.382), p = 0.025) (Table 3).

Table 3. Association between ALK mRNA expression level with histological subtype and immunohistochemical protein expression.

	OR	**95% CI (*p*-Value)**
Histological classification *Reference category: Group B*	1.914	1.083–3.382 (0.025)
Weak immunohistochemical protein expression *Reference category: negative protein expression*	2.829	1.290–6.204 (0.009)
Moderate/strong immunohistochemical protein expression *Reference category: negative protein expression*	2.934	0.889–9.679 (0.077)

Abbreviations: ALK, anaplastic lymphoma kinase; CI, confidence interval; and OR, odds ratio.

3.3. mRNA Expression and Immunohistochemical Protein Expression of ALK

The median CPM for tumors with negative ALK staining was 89 CPM (n = 33), for weak ALK staining 239 CPM (n = 11) and for moderate/strong ALK staining 244 CPM (n = 4).

The association between ALK mRNA expression and immunohistochemical protein expression is shown in Table 3. Tumors with weak ALK staining have higher gene expression levels of ALK than tumors with negative staining (OR 2.829, 95% CI (1.290–6.204), p = 0.009). This association is stronger when comparing samples with moderate/strong staining with those that show no ALK positivity, but are not significant (OR 2.934, 95% CI (0.889–9.679), p = 0.077).

3.4. ALK Status and mRNA Expression of ALK

Data on mRNA gene expression levels were only available for one ALK-amplified neuroblastoma. Therefore, no comparison could be made between mean the mRNA expression level of ALK and the ALK amplification status. This neuroblastoma showed an mRNA expression level of 5185 CPM, which was the highest expression level detected in this cohort. No rearrangements in the ALK gene were detected in this ALK-amplified neuroblastoma, or in any ALK non-amplified neuroblastomas.

A gain of either the whole chromosome 2 or the whole chromosome arm 2p was present in 18/53 neuroblastomas in which copy number analysis was performed (either SNP-array or WES). There is no significant difference between the mean ALK mRNA expression level of neuroblastomas with a gain of chromosome(arm) 2(p) (138 CPM) and the level of wild type chromosome 2 (192 CPM) (p = 0.129).

There is a significant difference between mean mRNA expression level of neuroblastomas with an ALK mutation (233 CPM, $n = 7$) and mean mRNA expression level of neuroblastomas without an ALK mutation (164 CPM, $n = 45$) ($p < 0.001$).

3.5. MYCN Status and mRNA Expression of ALK/MYCN

There is a significant difference between the MYCN mRNA expression level in MYCN-amplified neuroblastomas (750 CPM, $n = 11$) and mean MYCN mRNA expression level of MYCN non-amplified neuroblastomas (36 CPM, $n = 40$) ($p < 0.001$).

However, there is no significant difference between mean ALK mRNA expression level in MYCN-amplified neuroblastomas (174 CPM, $n = 11$) and mean ALK mRNA expression level of MYCN non-amplified neuroblastomas (177 CPM, $n = 40$) ($p = 0.553$).

4. Discussion

This study examined the relationship between ALK mRNA expression, OS and EFS, immunohistochemical protein expression, histological subtype, ALK aberrations (both mutations and amplifications) in neuroblastomas. We expected mRNA expression levels to be higher in ALK-mutated and ALK-amplified tumors. Furthermore, we hypothesized that ALK mRNA expression levels would be associated with a less differentiated histological subtype and immunohistochemical protein expression. Both hypotheses proved to be true. We showed that mRNA expression levels of ALK are indeed higher in ALK mutated than in non-mutated neuroblastomas. This is in line with previous studies, and it also explains why knockdown of ALK mRNA results in neuroblastoma cell lines, and results in growth inhibition of mutant and amplified neuroblastoma cells [3,25,53]. The mechanism behind this remains unclear [3]. Possibly mutant ALK genes promote their own expression via a feed-forward regulatory mechanism, similar to the feed-forward loop in ErbB2 expression in breast cancer [54]. However, there are numerous mechanisms by which mutations affect mRNA synthesis and these mechanisms differ from gene to gene [55].

This is the first study to establish the association between high ALK mRNA expression level with higher ALK immunohistochemical protein expression, though this relationship is moderate [56]. It is likely that no strong association was found because of differential translation of mRNA to protein and differential splicing of mRNA [19–21]. Furthermore, ALK mRNA expression levels are higher in neuroblastomas with unfavorable histology. This might be due to inhibition of DLG2 which would normally inhibit tumor growth and drive differentiation. ALK activation blocks this differentiation, which explains why neuroblastomas with high ALK mRNA expression levels are more often undifferentiated or poorly differentiated tumors [31].

Although RNA sequencing was only performed in one ALK-amplified neuroblastoma, the RNA profile of this poorly differentiated neuroblastoma was noteworthy, since its ALK mRNA expression level was 33 times higher than the median expression level of the other undifferentiated and poorly differentiated neuroblastoma. Other studies also reported extremely high expression levels in ALK-amplified neuroblastomas [37]. Furthermore, in MYCN-amplified neuroblastomas, the MYCN mRNA expression level is indeed higher than in MYCN non-amplified neuroblastomas. This supports our hypothesis that amplification of a gene results in a higher mRNA expression level. However, gain of whole chromosome 2 or chromosome arm 2p does not result in higher ALK mRNA expression levels. This might be due to variations in tumor percentage per tissue sample, since expression levels are correlated with tumor purity [57].

Whole transcriptome sequencing is a relatively new technique and limited studies on ALK mRNA expression have been performed. However, the results of this study suggest that whole transcriptome sequencing could be a promising tool in detecting both ALK amplifications, ALK mutations and predicting ALK immunohistochemistry positivity in patients with a neuroblastoma. To implement our findings in clinical practice, we would need to establish cut off values for mRNA expression levels. High mRNA expression levels would indicate ALK positive immunohistochemical protein expression and ALK mutation,

whereas extremely high levels suggest a strong immunohistochemical protein expression and an ALK-amplified neuroblastoma.

Previous studies found ALK gene expression to be associated with poor outcome [3,35,36]. Contrary to these studies, the results of our study suggest no relationship between mRNA expression of ALK and both OS and EFS. However, based on our data, MYCN amplification was also found not to be related to inferior outcomes, while MYCN amplification has proven to be the most important independent predictor of poor outcome [58]. Nevertheless, our findings do show a strong trend towards a correlation between MYCN amplification and EFS. We expect that MYCN amplification would have correlated with prognosis if our study population would have been larger. This small number of patients included is the most important limitation of the study. For this reason, no multivariate logistic regression model with MYCN and age group as possible confounders was performed. For the same reason, no multivariate Cox model was executed. As previously mentioned, the small sample size might also be the reason why both ALK gene expression and MYCN amplification were not regarded as risk factors for the poor outcome.

5. Conclusions

Contrary to previous studies, our study did not establish a significant relationship between high ALK mRNA expression and poor outcome. However, it did show an association between ALK mRNA expression and both ALK immunohistochemical protein expression and histological subtype of neuroblastomas. Furthermore, mRNA expression levels are higher in ALK-mutated neuroblastomas and seem to be much higher in ALK-amplified neuroblastomas too. Also, MYCN-amplified neuroblastomas have higher MYCN mRNA expression levels, but do not have higher ALK mRNA expression levels. More research, with a larger study population and a longer follow-up time, is needed to establish the prognostic relevance of ALK gene expression. Should this be confirmed in larger studies, inclusion into diagnostic procedures and classification systems should follow. Furthermore, cut-off values for mRNA expression are needed to be able to implement our findings in clinical practice. In the future, we might be able to use whole transcriptome sequencing as a technique to determine whether a neuroblastoma is ALK amplified, carries an ALK mutation and to predict ALK immunohistochemistry positivity in patients with a neuroblastoma. This information may be used to decide which patients are eligible for receiving ALK TKIs.

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