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Identification of *ALK* Mutation in Neuroblastoma on the Point of Molecular Heterogeneity

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Abstract

Background and Aim: In neuroblastoma, anaplastic lymphoma kinase mutations have recently received attention as molecular targets for the treatment of neuroblastoma, as 6% to 10% of patients with neuroblastoma have anaplastic lymphoma kinase mutations. There are little data from the cases in Turkey. We aimed to detect anaplastic lymphoma kinase mutations and molecular heterogeneity in neuroblastoma using next-generation sequencing. This study is the first one with this many cases in Turkey. **Methods:** Next-generation sequencing analysis was performed using an Illumina MiniSeq custom gene panel. Clinically important mutations were selected for the analysis. We also gathered clinical data of the patients from Turkish Pediatric Oncology Group cohorts to associate them with anaplastic lymphoma kinase mutations. This study is a retrospective cross-sectional study. We followed STROBE guideline (https://www.equator-network.org/reporting-guidelines/strobe/) on this study. **Results:** We analyzed anaplastic lymphoma kinase in 108 patients with neuroblastoma, with a mean age of 43.76 months. Pathogenic anaplastic lymphoma kinase mutations were detected in 13 patients (12.04%). We noted that anaplastic lymphoma kinase mutations were primarily observed in intermediate- and high-risk patients ($P = .028$). R1275Q and F1174-related mutations were predominant; I1171T, L1226F, S1189F, V1135A, and G1125S mutations were rare. Duplicate samples did not exhibit any heterogeneity. **Conclusions:** We found that F1174 and R1275Q-related anaplastic lymphoma kinase mutations are the most common pathogenic mutations in neuroblastoma. Anaplastic lymphoma kinase mutation status did not show any heterogeneity, and the mutations were correlated with intermediate- or high-risk groups.

Keywords

neuroblastoma, *ALK* mutation, heterogeneity, next-generation sequencing, high-risk groups

Abbreviations

ALK, anaplastic lymphoma kinase; FISH, fluorescence in situ hybridization; INRG, International Neuroblastoma Risk Group; MYCN, neuroblastoma-derived V-Myc avian myelocytomatosis viral related oncogene; NB, neuroblastoma; NGS, next-generation sequencing; OS, overall survival; PCR, polymerase chain reaction; PFS, progression-free survival.

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Introduction

Recent advances in targeted therapies are becoming increasingly important for the treatment of patients with cancer, particularly for pediatric patients. Patients with higher-stage and high-risk neuroblastoma (NB) have a worse prognosis than low-risk patients as the 5-year overall survival rates in high-risk and low-risk patients are 40% to 50% and >90%, respectively.[1,2]

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NB is a heterogeneous disease characterized by various chromosomal rearrangements, such as *MYCN* gene amplification (24% of all cases), partial deletions in chromosomes 1p and 11q, partial additions in chromosome 17q, and triploidy.[3] Management of relapse in high-risk and refractory NB cases is difficult; therefore, novel treatment strategies are needed. Anti-GD2 immunotherapy and therapies directed against tumor-specific molecular alterations, such as anaplastic lymphoma kinase (ALK), increase survival of patients with high-risk NB.[2] Combination of chemotherapy, radiotherapy, and stem cell transplantation is other treatment options. Isotretinoin combined with anti-GD2 immunotherapy and targeted kinase inhibition is a potential treatment strategy for patients with high-risk NB.

Different kinases—such as ALK, aurora kinase, and RET receptor tyrosine kinase—are potential therapeutic targets for various cancers.[4] ALK acts as an oncogenic driver in cancer; therefore, *ALK* gene is one of the major targets for inhibition in NB. *ALK* encodes a tyrosine kinase receptor belonging to the insulin receptor superfamily. It is expressed in the developing central and peripheral neural tissues, where it plays a supporting role in embryonic nervous system development; however, *ALK* expression decreases after birth. Neuroblastoma likely develops due to malignant transformation of partially committed sympathoadrenal neuroblasts, during fetal or early childhood development.[5]

ALK gene is found at chromosome band 2p23; it has 29 exons. The extracellular ligand-binding, transmembrane, intracellular juxta membrane, and kinase domains of this gene comprise 1620 amino acids. The tyrosine kinase domain exists between 1116 and 1392 amino acids.[6,7] *ALK* is activated in cancer via different mechanisms. Anaplastic large-cell lymphoma, inflammatory myofibroblastic tumors, nonsmall cell lung cancer, and breast, colon, and renal cancers show the t(2;5) (p23;q35) chromosomal translocation. This translocation produces a fusion protein called nucleophosmin which acts as an oncogene. In NB, various point mutations or gene amplifications are observed.[6–9] Genetic alterations in *ALK* often lead to constitutive activation of the tyrosine kinase domain. In wild-type ALK, the kinase domain exists in an auto-inhibited conformation; the ALK receptor dimerizes and the tyrosine kinase domain is phosphorylated. This activates the MAPK, PI3K, or JAK/STAT pathways.

ALK mutations occur in the germline in 50% of familial NB cases, and only in 15% of somatic NBs. Mutations in germline cases are inherited in an autosomal dominant manner.[7,8] Patients with NB having both *MYCN* amplification and *ALK* mutations show poor prognosis. Patients receiving crizotinib or second-generation ALK inhibitors, such as alectinib and lorlatinib as targeted therapy, might receive survival benefits after selection of the *ALK* mutation type using next-generation sequencing (NGS).[9]

In NB, mutations at 3 key positions, namely F1174, F1245, and R1275, account for approximately 85% of *ALK* mutations. R1275Q is the most common type of mutation observed in about 45% of familial cases and a third of sporadic cases, whereas F1174 and F1245 mutants are exclusively found in sporadic NB cases, at frequencies of approximately 30% and 12%, respectively.[7–9] Interestingly, the Y1278S mutation observed in patients with NB harbors gain-of-function activity.[3] The aim of this study is to investigate ALK mutation heterogeneity in NB cases in Turkey by NGS.

Materials and Methods

Ethical approval was obtained from the Local Human Non-invasive Studies Ethics Committee of our university (approval no.: 2018/05-21; date: 15.02.2018).

Patients and Samples

This study included 108 patients diagnosed with NB in Turkey between 2012 and 2020. Written informed consent was obtained from all participants. The patients enrolled in this study showed 2 different somatic tumor tissues: either simultaneously or different tissues from the initial diagnosis and relapsed/metachronous metastatic tissues. The patients were diagnosed, treated, and followed up according to the International Neuroblastoma Risk Group (INRG) Classification System[10] revised in 2021. Our goal is to describe the ALK mutation profile. All cases that were studied for ALK were included.

In routine procedures, *MYCN* amplification, 11q23 deletion, and DNA ploidy were assessed as previously described.[11] Modified Shimada classification was used to determine pathologically favorable or unfavorable histology, based on age, tumor differentiation, and mitotic karyorrhexis index. Anaplastic lymphoma kinase break-apart FISH (fluorescence in situ hybridization) was performed to detect possible chromosomal translocations. Fresh frozen tissues stored at −80 °C were used for NGS.

Preanalytic Procedures

Freshly frozen tumor tissues (3 mm^3) from each pair of samples were used for DNA isolation, using the High Pure PCR Template Preparation Kit (11796828001; Roche) according to the manufacturer's instructions. DNA was quantified using a Qubit™ dsDNA BR (Broad-Range) Assay Kit (Q32850; Invitrogen). Samples having DNA more than 15 µg/mL were selected for further NGS analysis. Patients with *ALK* mutations were recalled, and peripheral blood was obtained for germline mutation studies. NGS with the same panel was applied to DNA extracted from peripheral blood mononuclear cells. DNA from 4 frequently used NB cell lines—Kelly, SHSY5Y, LAN5, and CHP134—were also analyzed by NGS.

Next-Generation Sequencing Analytic Procedures

A custom-designed NGS kit based on target capture was used for analysis (Neuroblastoma Panel, NEUR 01 AD16; Celemics, Inc.). The "neuroblastoma" custom sequencing panel was designed to cover all regions of *ALK* and other genes in the

kit. The prepared NGS gene list was similar to the custom panel previously described by Chen et al which was designed in collaboration with the SIOPEN Biology Groups.[6,7]

DNA was fragmented to 150 to 250 base pairs at 37 °C using a dsDNA Fragmentase kit (Cat No CM0816; Celemics, Inc.). Purification was performed in-between each step using CeleMag Clean-up Beads (NEUR 01 AD16; Celemics, Inc.). A-tailing was performed on the DNA fragments by adenylation. Adaptor ligation, index labeling, probe hybridization, and library preparation were done using CeleMag Streptavidin Beads (Celemics, Inc.), and this was followed by PCR amplification of target library in thermal cycler overnight at 65 °C (Library Prep Kit; KAPA Library Amplification Mix; Target Capture Solution Box; Celemics, Inc.). The indexed library prepared using the target capture NGS kit was measured using a Qubit for each case. All samples were gathered in one tube after calculations using the same constraints. The final library was loaded onto a MiniSeq™ High Output Reagent Cartridge (Ref 15073286; Illumina, Inc.); the cartridge and flow cell (MiniSeq™ Flow Cell; Ref 15073187; Illumina, Inc.) were loaded onto an Illumina Miniseq system (Illumina, Inc.,) for sequencing with paired-end reads. FASTQ data were obtained after 16 h. The genomic coordinates were extended by 50 bp upstream and downstream to obtain a final target of approximately 729 Kb for *ALK*.

Post-Analytic Bioinformatic Procedures

The reference genome used to map the NB samples was obtained from Ensembl VEP, version 78 (https://github.com/Ensembl/ensembl-vep/releases). The tools used for data processing were Burrows–Wheeler Aligner for alignment[12] and Freebayes for variant calling.[13] We annotated using Ensembl and kept the variants with the following criteria: (a) protein sequence impacting, (b) with allele frequency lower than 0.05 in human population databases, (c) not registered in dbSNP version 151, and (d) without sequencing strand bias. Mutations between amino acids 1116 and 1383 that encode tyrosine kinases were taken into consideration with clinically important mutation loci.

Statistical Methods

The *ALK* mutation as an independent variable was recorded as mutation present or absent in tyrosine kinase domain (F1174, R1275, or other). The staging, risk group, and diagnostic variables were done according to TPOG 2012 and TPOG 2020 protocol. Clinical and laboratory data, such as patient age, sex, risk category, and *MYCN* amplification status, were statistically evaluated by nonparametric tests using the Mann-Whitney *U* test. Statistical significance was set at $P < .05$. Survival analyses were not performed because of missing data. Cases with missing data are included. We used STROBE guidelines for cross-sectional studies (https://www.equator-network.org/reporting-guidelines/strobe/).

Results

Patient Characteristics

ALK was analyzed in 108 patients; 59 cases were male (54.6%) and 49 were female (45.4%). Since ALK was our only criteria, all patients were eligible. Mean age was 43.76 ± 40.28 (1-192) months. Age of ALK mutated cases was 78 ± 66.3 months (of patients that do not have mutation 30.77 ± 34.71 months, *t* test $P = .112$). In 7 cases (7/115, 6.09%), DNA quantification was not satisfactory. These cases were excluded from the study, and NGS was not performed.

MYCN was amplified in 28 (25.9%) patients; 42 cases (40.8%) harbored an 11q23 deletion. 65 cases were high risk (60.2%), 23 (21.3%) were intermediate risk, and 20 (18.5%) were low risk. Among the 13 cases with a patent *ALK* point mutation, *MYCN* was amplified in 30.8%, 11q deletion was observed in 53.8%. All ALK mutated cases were intermediate risk (46.2%) or high risk (53.8%). Of these patients, 57.1% had locoregional disease and 42.9% had metastatic disease in ALK mutated cases. None of the cases showed break-apart locations in FISH. Two cases showed amplification of ALK in FISH analysis.

Next-Generation Sequencing ALK Mutation Results

Pathogenic *ALK* mutations were detected in 13 patients (12.04%). *ALK* mutations were observed in intermediate- and high-risk patients ($P = .028$). Four patients with both somatic and germline R1275Q mutations belonged to the same family. One patient harbored a nonfamilial R1175Q mutation. The F1174L mutation was observed in 4 cases: one case had the F1174C mutation and another had the F1174Q mutation. One patient harbored an I1171T mutation. One patient had multiple mutations, including L1226F, S1189F, V1135A, and G1125S. In 65.74% of the 108 cases, the I1461V, K1491R, and D1529E mutations with high allele frequencies were observed that are not of clinical importance. In 44 cases, double samples were studied to explore molecular heterogeneity. Synchronous samples from different areas of the tumor were studied in 26 cases. Metachronous tumors were studied at different time points in 18 cases. There was no case of *ALK* mutation in only one patient. The same mutation was observed in all 44 double samples. Only in one case, 4 more *ALK* mutations without clinical importance was observed. The mutation sites and frequencies in relation to patient characteristics are shown in Table 1. In the survival analysis, we did not observe any association between *ALK* mutations or loci and clinical outcomes (including progression-free survival and overall survival). The NGS datasets generated and analyzed in this study are available from the corresponding author upon request.

Among cell line samples, Kelly and SHSY5Y cells showed F1174L point mutations while LAN5 and CHP134 cells not. All 4 cell lines harbored an I1461V benign *ALK* mutation, which is common in NBs.

Table 1. Patient Characteristics of the cases with ALK Mutation.

No.	Age (in months)/sex	Risk	*ALK* mutation	*MYCN* amplification	11q23 deletion	DNA ploidy	Shimada classification
NB 704	8/Female	Intermediate	R1275Q somatic + germline	Negative	Negative	Triploidy	Unfavorable
NB768	14/Female	Intermediate	R1275Q somatic + germline	Negative	Negative	Tetraploidy	Favorable
NB1235	120/Male	Intermediate	R1275Q, somatic + germline	Negative	Positive	Triploidy	Favorable
NB595	8/Female	Intermediate	R1275Q, somatic + germline	Negative	Negative	Triploidy	Unfavorable
NL 63	6/Male	High	R1275Q, somatic	Positive	Negative	Diploidy	Unfavorable
NL224	156/Female	High	F1174Q somatic	Negative	Positive	Diploidy	Favorable
NL84	31/Male	High	F1174L somatic	Negative	Negative	Diploidy	Unfavorable
NL247	13/Male	High	F1174L somatic	Positive	Positive	Diploidy	Favorable
NL220	43/Female	High	F1174L somatic	Positive	Positive	Hipoploidy	Unfavorable
NB1387	12/Male	High	F1174L somatic	Positive	Positive	Diploidy	Unfavorable
NB1303	60/Female	High	F1174C somatic	Negative	Positive	Diploidy	Unfavorable
NL154	36/Female	Intermediate	I1171T somatic	Negative	Negative	Diploidy	Unfavorable
NL241	132/Female	High	L1226F, S1189F, V1135A, G1125S somatic US	Negative	Positive	Diploidy	Unfavorable

Abbreviations: NB, neuroblastoma; ALK, anaplastic lymphoma kinase; MYCN, neuroblastoma-derived V-Myc Avian myelocytomatosis viral related oncogene; US, undetermined significance.

Discussion

In this study, we performed *ALK* gene sequencing using NGS in paired samples of NB tumor tissues to explore molecular heterogeneity. The samples were obtained from different parts of the tumor at the same time, and from primary diagnostic and tumor tissues during relapse of the same patient at different times. We did not observe molecular heterogeneity in *ALK* mutations between the 2 samples in either group.

ALK is only phosphorylated in developing central and peripheral nerve tissues. These mutations are overexpressed in cancer.[9,14] Although *ALK* oncogene activation increases in many human cancers, such as lung carcinoma and fusion proteins (NPM-ALK fusion in anaplastic large cell lymphoma; EML4-ALK in nonsmall cell lung cancer) through chromosomal translocation events,[15] more than 20 point mutations in the conserved regions of the tyrosine kinase domain have been identified in NB.[6] Fusion proteins lead to ligand-independent self-dimerization, autophosphorylation, and transphosphorylation of ALK. Point mutations cause autophosphorylation. *ALK* has been identified as a potential pharmacological target in NB when ALK knockdown results in growth inhibition in all NB cell lines with *ALK* mutations and some with wild-type ALK.[16]

The INRG classification has led to 16 statistically distinct risk groups based on clinical and molecular features, which has made prognosis more accurate for patients and helped guide physicians in treatment regimens. In Turkey, the NB protocol based on the INRG is used as a national protocol, supported by the Turkish Pediatric Oncology Group Association. Detection of *ALK* and other genes by NGS for targeted therapy decisions has not yet been included in the protocol. However, it is currently used to determine new therapeutic approaches in relapsed and refractory cases. Neuroblastoma cases, including those with the R1275Q mutation in *ALK*, are sensitive to first-generation ALK inhibitors, whereas those with F1174 mutations are resistant. Patients with F1174 mutations are sensitive to second-generation ALK inhibitors.[17] *ALK* amplification is not an indication for targeted therapy in NB. In one of the 2 cases where ALK amplification was detected ALK F1174 mutation was also present. The other case that only had ALK amplification was not considered for targeted therapy.

ALK mutations were found in 12.4% of cases. Donohue et al analyzed 641 cases using whole-genome sequencing or hotspot *ALK* mutation profiling and found *ALK* mutations in 16% of cases. Approximately 10% of all NB harbor somatic *ALK* mutations at diagnosis (with reports of even higher incidence at relapse), 85% of which are accounted for by single nucleotide variants at 3 loci: R1275 (43%-49%), F1174 (30%-35%), and F1245 (12%).[7] *ALK* mutations are present in 8% to 14% of all NB at diagnosis, increasing to 26% to 43% at relapse. Allinson et al reported 2 cases of loss of *ALK* mutations during relapse (*ALK* F1174Land *ALK* R1257Q).[18–20]

In the present study, patients with somatic F1174 mutations were treated with lorlatinib. The patients were clinically good for one year. Follow-up was continued. One case with F1174L mutations showed 16% of the same mutation in peripheral blood. However, this was considered to be circulating DNA instead of germline mutations. This does not apply to homozygous or heterozygous mutations. We interpreted this germline mutation was from circulating DNA but could not confirm it.

Characterization of the different point mutations in *ALK* observed in NB patients has led to the segregation of mutations into 3 classes: ligand-independent, ligand-dependent, and kinase-dead forms of receptor.[3] The tyrosine at positions 1158, 1278, 1162, and 1163 is important for autophagy. In the present study, mutations in the kinase domain were recorded. The first 4 cases shown in Table 1 are from the same family. There are cousins. There was one more case in the same family history of death from NB in which we could not reach the tumor tissue or blood. The R1275Q point mutation was detected as expected.[21–23] The ALK-mutated NB cases in

our series were not found to be related to *MYCN* amplification. *MYCN* was not amplified in familial cases with germline R1275Q mutations. We think that this is the reason our results did not associate ALK with *MYCN* amplification. Most cases of *ALK* mutations have been reported to be amplified by *MYCN*.[24] When we exclude familial cases the ratio of *MYCN* amplification is 4/9 (44.44%). This is higher than NB population but the number of cases is too small to perform meaningful statistical analysis.

The weakness and limitations of the study are that the total number of cases in which *ALK* mutations were studied was less than that in the literature. Another weakness is the selection bias caused by the demand from clinicians since we are only studying ALK on their demand. This may be the main reason that our mutation profile differs from other cohorts. We plan to add ALK to routine clinical diagnosis to prevent selection bias. The strength of this study is in the number of the samples. Another strength of our study is that we performed 2 sample analyses for many cases. We report F1174L mutation in germline. To our knowledge, this is the first study to evaluate the differential impact of various ALK mutation loci on NB in Turkey.

Conclusion

In this study, we aimed to identify the most common *ALK* mutations in NB but did not observe any somatic heterogeneity in *ALK* mutation status in different spatial and temporal samples of patients with NB. We concluded that it is better to study NGS in the initial biopsy of a patient, regardless of whether it is primary or metastatic. The surgical material after neoadjuvant chemotherapy was unsatisfactory. These samples included large areas of treatment effects such as necrosis, fibrosis, hemorrhage, Schwannian, and gangliocytic differentiation. The relapse of tumor tissue, if satisfactory areas of blastic tumors are present, may be used. Bone marrow biopsy is not useful for obtaining high-quality DNA samples. Bone marrow aspiration followed by MACS or FACS selection of GD-2 positive cells by anti-GD-2 antibody might be attempted if more tumor tissue is available for NGS.

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Authors' Note

Ethical permission was taken from the Local Human Noninvasive Studies Ethics Committee of Dokuz Eylül University (Date: 15.02.2018, no: 2018/05-21).

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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