

Original Research

Unraveling the Mystery: Next Generation Sequencing Sheds Light on Neuroblastoma Pathogenesis and Targeted Therapies

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Abstract

Background: There is considerable interest in the molecular evaluation of solid tumors in pediatric cases. Although clinical trials are in progress for targeted therapies against neuroblastoma (NB), novel therapeutic strategies are needed for high-risk cases that are resistant to therapy. The aim of the present study was to document the specific gene mutations related to targeted therapy in relapsed or refractory NB patients by using next generation sequencing (NGS). **Methods:** The study included 57 NB patients from amongst 1965 neuroblastic cases in Turkey who experienced a recurrence after multi-model therapy. The cases were diagnosed, risk-stratified, and treated according to the classification system from the International Neuroblastoma Risk Group. Single nucleotide variations in 60 genes were investigated using the Pillar Onco/Reveal Multicancer v4 panel and Pillar RNA fusion panel on the Illumina Miniseq platform. **Results:** ERBB2 I655V was the most frequent mutation and was found in 39.65% of cases. Anaplastic Lymphoma Kinase (ALK) mutations (F1174L, R1275Q, and rare mutations in the tyrosine kinase domain) were detected in 29.3% of cases. Fusion mutations in NTRK1, NTRK3, ROS1, RET, FGFR3, ALK and BRAF were observed in 19.6% of cases. **Conclusions:** This study presents valuable mutation data for relapsed and refractory NB patients. The high frequency of the ERBB2 I655V mutation may allow further exploration of this mutation as a potential therapeutic target. Rare BRAF mutations may also provide opportunities for targeted therapy. The role of ABL1 mutations in NB should also be explored further.

Keywords: neuroblastoma; mutation; high-throughput nucleotide sequencing; therapy; anaplastic lymphoma kinase; erbb-2; BRAF-KIAA1549 fusion protein; DNA mutational analysis

1. Introduction

Cancer involves the uncontrolled division of cells with immortal proliferative capacity. The tumor cells acquire genomic instability and an increasing number of mutations during each division, which allows them to evade cell cycle control, escape immune destruction, and form a suitable “niche” microenvironment with increased vasculature. Finally, the acquisition of invasive and metastatic properties leads to cancer becoming a systemic disease with serious clinical consequences. In addition to advances in early detection, surgical resection, chemotherapy and/or radiotherapy strategies, more recent developments involve targeted therapies based on comprehensive analysis of gene mutations [1].

The incidence of neuroblastoma (NB) is 11–13 cases per million children aged <14 years. In children aged <12 months, the incidence of NB is 65 cases per million according to SEER databases. Survival from NB is much better in younger children [2], and this cancer type is very rare in adulthood.

NB is a neural embryonal cancer of early childhood that arises from sympathetic ganglion cells derived from neural crest cells. These cells migrate from the neural tube during early embryogenesis [3–6]. In children, NB originates from the adrenal medulla or from cells of the sympathetic nervous system on both sides of the spine (paravertebral ganglia). Palpable swelling in the abdomen, significant bruising around the eyes, and various other clinical signs and symptoms can be observed depending on the origin of NB. When NB develops on both sides of the spine, it can cross the vertebrae and apply pressure to the spinal cord, leading to conditions such as loss of strength or complete paralysis in the legs, as well as urinary or fecal incontinence. NB shows considerable heterogeneity in clinical, pathologic and molecular properties. To assess its tumor burden, biology and prognosis, NB is categorized into very low, low, intermediate, high and very high risk categories. Patient age, tumor stage (L1, L2, M, and MS), surgical risk factors, imaging results, grade of tumor differentiation, and biomarkers (N-Myc amplification, 17q gain, 1p deletion,



11q deletion, DNA ploidy) are used in risk assessment [7,8]. The International Neuroblastoma Risk Group (INRG) staging system was first proposed by Cohn and Pearson and updated by the Children's Oncology Group (COG) [9–12].

Many genetic and epigenetic events occur during neural crest maturation and migration from the neural tube to primordial neuroectoderm, and then to mature ganglia or adrenal medulla. These include genetic alterations to *ZIC1*, *PAX3*, *TPAP2a*, *Notch*, *PRDM1A*, *SOXE* family genes, *ZEB2*, *BMP*, *Wnt*, *FGF*, *FOXD3*, *C-Myc*, and *N-Myc*. Gain of 17q, loss of 1p, and loss of 11q are the most common genetic changes, while *N-Myc* amplification (20–25% frequency) is a biomarker for advanced disease. Functional molecular oncology approaches have recently been used in an attempt to link clinicopathologic properties with specific molecular changes [13–16].

The various therapies for NB include surgical removal of the tumor mass, chemotherapy, radiotherapy, immunotherapy, and autologous stem cell transplantation. Despite some success in managing this disease, approximately half of NB cases are high-risk and the 5-year survival rate is <40%. Multi-agent chemotherapy has been used in high-risk cases [17,18]. Chemotherapy combinations are also used for relapsed cases, some of which become refractory to treatment and hence the patient becomes prone to disseminated metastatic disease. In such cases, immunotherapy and/or targeted therapies may improve patient survival.

The number of targeted drugs approved by the FDA is quite limited, especially for NB and pediatric tumors. Treatments targeted to 43 gene changes were approved in 28 different tumor types, most of which are adult cancers. Current targeted therapy trials for high-risk NB include the Anaplastic Lymphoma Kinase (ALK) inhibitors Crizotinib and Lorlatinib. The R1275Q mutation in ALK is sensitive to first generation ALK inhibitors, whereas patients with F1174 mutations are resistant. However, patients with F1174 mutations are responsive to second generation ALK inhibitors [19,20]. The aurora kinase inhibitor Alisertib (MLN8237) is also currently being tested for NB in combination with chemotherapy. This drug indirectly disrupts *N-Myc* to induce cell cycle arrest, apoptosis, and autophagy. Other agents being tested for NB include the Raf inhibitor Sorafenib, PI3K inhibitors that block the RAS pathway, and the anti-VEGF bevacizumab [13]. Immunotherapy with the monoclonal anti-disialoganglioside GD-2 has also been shown to improve the survival of high-risk NB patients [10]. The most difficult scenario encountered during the treatment of cancer patients is the lack of response to any treatment. In such cases, the detection of a targetable mutation may provide the final treatment opportunity. Health insurance companies do not usually cover this treatment, although permission to use off-label drugs can be requested.

Most targeted therapy agents require molecular assessment to provide evidence for indication. The detec-

tion of somatic variants in tumor tissue by high throughput sequencing has been used in clinical practice to identify predictive markers, as well as for diagnostic or prognostic purposes. The investigation of some molecular targets is case-specific, for example the assessment of mutation status for a specific targeted therapy according to cancer and organ subtype. Some targeted therapy inhibitors are agnostic, such as the neurotrophic tyrosine kinase (NTRK) inhibitors. Hence, the detection in any tumor of NTRK fusion leading to increased tyrosine kinase activity provides indication for the use of NTRK inhibitors.

Next-generation sequencing (NGS) allows the detection of rare individual gene mutations [21]. Although somatic mutations are infrequent in NB, they have been observed in a few genes such as ALK [22,23]. NB is generally referred to as a “cold” tumor type because it has a very low mutation burden. The NGS method is now used routinely for molecular evaluation of solid tumors in adults. It is also increasingly being used for pediatric cancers [24,25]. cDNA is obtained from RNA by reverse transcriptase and then sequenced by NGS. This method is becoming widely used, especially for the evaluation of translocations. Although still expensive, RNA analysis for translocations is especially important for diagnosis and for the selection of targeted therapy in soft tissue, central nervous system and salivary gland tumors [26].

Each year about 3000 cases of childhood cancer are diagnosed in the Turkish Republic, of which around 300 are NB. Half of these NB cases are metastatic at diagnosis. Increased knowledge of NB at the molecular level is needed to better understand its pathogenesis and to develop novel therapeutics that could improve outcomes in high-risk, relapsed, or refractory cases. The aim of the present study was therefore to present targeted NGS data for somatic mutations in a Turkish cohort of relapsed and refractory NB cases.

2. Materials and Methods

Targeted, capture-based NGS was performed on 60 selected cancer-related genes. This was designed to explore targeted therapy options for relapsed and refractory NB patients. DNA was extracted from tumor tissues that were freshly frozen and stored at -80°C , or from formalin-fixed and paraffin-embedded (FFPE) tumor tissue from diagnostic archival tissue. RNA was also extracted and converted to complementary DNA for the detection of 87 fusions in 18 genes.

2.1 Patient Characteristics

The patient cohort comprised 57 NB cases from a series of 1965 neuroblastic tumors from the Turkish Pediatric Oncology Neuroblastoma Study Group. The enrolled patients were relapsed or refractory NB cases that did not respond to salvage therapies. Some patients received anti-GD2 therapy, the results of which will be reported else-

where. NGS was used here with the aim of finding options for targeted therapy. Patients were staged and risk-categorized according to the INSR scheme. Molecular tests for N-Myc, 11q deletion and DNA ploidy were performed by a molecular pathologist and molecular oncologist at a specialist center in our institute [27,28]. Shimada classification was carried out by trained pathologists.

2.2 Sample Collection

Fresh frozen tumor samples stored at -80°C were the preferred tissue source for DNA and RNA extraction. FFPE tumor tissue was used if fresh frozen tissue was not available. Tissue samples (3 mm^3) for DNA and RNA isolation were collected in two separate microtubes. DNA isolation, RNA isolation and cDNA conversion were carried out according to the manufacturers' instructions (DNA Isolation Kit, version 23, N0: 11814770001, Lot: 37696800, Roche Diagnostics, Indianapolis, IN, USA; High pure RNA isolation kit, version 12, No: 11828665001, Lot: 14581300; Roche Diagnostics, Indianapolis, IN, USA; First Strand cDNA Synthesis Kit, version 09, No: 11483188001, Roche Diagnostics, Indianapolis, IN, USA; respectively). DNA quantification was carried out using the Qubit dsDNA High Sensitivity assay kit and a Qubit fluorometer.

2.3 Next Generation Sequencing

The Pillar ONCO/Reveal Multi-Cancer v4 commercial kit (REF: HRA-HS-1002-24, LOT 23PB0069, Pillar Biosciences, Natick, MA, USA) was used for SNV detection. The panel in this kit is comprised of 60 tumor-related genes (*ABLI*, *AKT1*, *ALK*, *APC*, *ATM*, *BRAF*, *CCNE1*, *CDH1*, *CDKN2A*, *CSF1R*, *CTNNA1*, *DDR2*, *EGFR*, *ERBB2*, *ERBB4*, *EZH2*, *FBXW7*, *FGFR1*, *FGFR2*, *FGFR3*, *FLT3*, *FOXL2*, *GNAI1*, *GNAQ*, *GNAS*, *HNFA1*, *HRAS*, *IDH1*, *IDH2*, *JAK2*, *JAK3*, *KDR*, *KIT*, *KRAS*, *MAP2K1*, *MET*, *MLH1*, *MPL*, *MYC*, *NOTCH1*, *NPM1*, *NRAS*, *NTRK1*, *NTRK2*, *NTRK3*, *PDGFRA*, *PIK3CA*, *PTEN*, *PTPN11*, *RAC1*, *RBI*, *RET*, *ROS1*, *SMAD4*, *SMARCB1*, *SMO*, *SRC*, *STK11*, *TP53*, *VHL*). Copy number variants were not analyzed.

PCR amplification (18 cycles) of genomic DNA targets was carried out using the Pillar Custom Indexing Primer Kit, CH222 Oligo Pool, Gene-Specific PCR Master Mix, GC Rescue G and 50 ng (10–75 ng) of DNA per PCR reaction. Uracil-DNA glycosylase (UDG) was added to avoid FFPE-related damage. Primer digestion was performed with exonuclease I. PCR product was purified using magnetic beads (Agencourt AMPure XP Beads) and washed with ethanol. Each PCR product was indexed with forward and reverse indexing primer adaptors and the indexing PCR Master Mix (Pi700 index and Pi500 index). Libraries were then amplified, purified, quantified, normalized and prepared for tracking on Illumina flow cells before sequencing with MiniSeq (High Output Reagent version 1,300 cycles, REF: 15073286, LOT: 20363687, Illumina,

Inc., San Diego, CA, USA) and Illumina (#MS-102-2002). Libraries were diluted to 5 nM prior to normalization and 5 μL of each diluted library was then added to each tube. After denaturing the 5 μL of library mix, 5 μL of 0.2 M NaOH and 990 μL of Illumina HT1 solution were added.

Next, 240 μL of the 25 pM library mix (step 5) was added to 360 μL of the Illumina HT1 solution. The library mix and PhiX control were combined and loaded onto the cartridge. Sequencing was performed using a paired-end read length of 150 bp after the sample sheet enters. Data output BCL (Binary Base Call) files were converted to FASTQ files using the bcl2fastq program. FASTQ files were aligned to a reference genome, namely the BAM (Binary Alignment Map) files, for the detection of variants [29–35].

The entire length of each gene, including introns and exons, was sequenced using this commercially available panel. The target capture-based sequencing approach was used to reveal the molecular profiles of all variant types. The commercial test used in this study employs stem-loop inhibition-mediated amplification technology to overcome problems with DNA damage present in FFPE tissues. This technology allows the study of poor quality DNA and/or low-input DNA [36].

2.4 RNA Fusion Detection

Selected regions in the RNA fusion panel were detected according to the manufacturer's instructions. The selected panels also included agnostic biomarkers such as NTRK1, NTRK2, and NTRK3. Regardless of the organ in which they are detected, this may provide options for treatment. An amplicon-based sequencing kit was used for RNA sequencing because it is more sensitive to specific genomic regions. The Pillar Onco/Reveal Multi-Cancer RNA Fusion v2 commercial kit (REF: HRA-HS-1002-24, LOT 23PB0069, Pillar Biosciences, Natick, MA, USA) was used to detect multiple gene rearrangement regions of interest in the *ALK*, *RET*, *ROS1*, *NRG1*, *PBX1*, *FGFR2*, *FGFR3*, *EGFR*, *NTRK1*, *NTRK2*, *NTRK3*, *BRAF*, *RAF1*, *MET*, *PPARG*, *ERG*, *PRKACA* and *TFE3* genes. These target driver genes and list of their fusion partners are given in **Supplementary file**. This assay uses Stem-Loop Inhibition-Mediated amplification technology. Tiled amplicon-based library preparation is applied to achieve efficient single-tube target enrichment.

2.5 Bioinformatics and Biostatistical Analyses

Data output BCL (Binary Base Call) files were converted to FASTQ files using the bcl2fastq program. FASTQ files were aligned to the reference genome, the BAM (Binary Alignment Map) files, thus allowing variant detection. Ensemble was used for annotation. Note was made of variants that: (a) impacted protein sequence, (b) had an allele frequency of <0.05 in human population databases, (c) were not registered in the dbSNP version 151, and (d) showed no sequencing strand bias.

Table 1. Summarization of the clinicopathologic information in patients of neuroblastoma.

Category	Number (n)	Statistics in Spearman Correlation
Number	(n = 57)	
Age (months) Median (Range)	48 (2–348 months)	$p > 0.05$
Sex	Male: 35; Female: 22	$p > 0.05$
Primary site	Adrenal: 31, Abdomen (nonadrenal): 12, Thoracal: 14	$p > 0.05$
Diagnosis	Neuroblastoma, differentiating: 4 poorly differentiated: 17 undifferentiated: 34 Ganglioneuroblastoma, intermixed type (GNB-I): 1 nodular type (GNB-N): 1	
International Neuroblastoma Pathology Classification	Favorable: 2, Unfavorable: 55	
Stage	L1: 3, L2: 5, M: 39	Risk: $p < 0.001$
<i>MYCN</i> status	Amplification: 15, No amplification: 42	Risk: $p = 0.018$
11q24 deletion	Deleted: 15, No deletion: 42	$p > 0.05$
DNA ploidy	DI = 1:38; DI>/<1:19	$p > 0.05$
Risk group at initial diagnosis	High risk: 48, Non-high risk: 9	
LDH (IU/L)	Median: 375 Range (247–3573)	Risk: $p = 0.021$
Ferritin (ng/mL)	Median: 59 Range (15–1295)	Risk: $p = 0.019$
NSE (ng/mL)	Median: 199 Range (17–500)	Risk: $p = 0.007$
VMA (mg/day)	Median: 116 Range (0.28–644)	$p > 0.05$
Death	(Mean 28 months follow-up) No: 55, Yes: 1	

L, Localized disease; M, Metastatic disease; LDH, Lactic dehydrogenase in serum; NSE, Neurone Specific Enolase in serum; VMA, vanillylmandelic acid in urine.

Clinical and laboratory data including patient age, gender, risk category, N-Myc amplification, 11q deletion, and DNA ploidy status were compared with the presence of mutations using non-parametric Chi-square test or Mann-Whitney U test. p -values < 0.05 were considered as statistically significant. Survival analysis was not performed here as the selected study cohort was relapsed or refractory.

3. Results

3.1 Patient Characteristics

The mean patient age at first diagnosis was 64.09 ± 62.83 months (range 2–348), with 61.4% of the cohort being male and 38.6% female. N-Myc amplification was detected in 26.3% of cases and 11q deletion in 25%. In addition, 66.67% of patients were diploid or near-diploid at first diagnosis. According to INSR, 84.21% of patients were high risk at diagnosis, 10.52% were intermediate risk, and 5.26% were low risk. The mean event time was 26 months. Clinicopathologic information for this patient cohort is summarized in Table 1.

3.2 Common Mutations

The DNA mutations detected by NGS are listed in Table 2. Intronic mutations were not recorded. Missense mutations, frameshift mutations and in-frame deletions were recorded, even if it was not known whether they are clinically important.

The true-positive mutation distribution for the study cohort is shown as an onco-plot in Fig. 1. The most common mutation was the ERBB2 I655V mutation, raising the question of whether it is a target in pediatric oncology. ALK mutations were also common. Besides the common F1174 mutations (e.g., F1174L) and R1275Q (especially in familial cases), rare ALK mutations were also found in the tyrosine kinase domain located in the intracytoplasmic region between amino acids 1116–1392 and including exon 23. The benign ALK I1461V mutation is not shown in the table as it is a common finding in all cases. The *ALK* gene is located at 2p23 and has 29 exons. The extracellular ligand-binding, transmembrane, intracellular juxta membrane and kinase domains for ALK are produced as a 1620 amino acid protein, with the tyrosine kinase domain located between amino acids 1116–1392 [22,23]. The *ABL1 M244V* gene mutation was found in 27.59% of cases and is located before the P-loop and near the N terminus. The role of this mutation in NB is not well defined. ATM, p53, RB1 and APC inactivating mutations are frequently reported to have a role in tumorigenesis. Various EGFR mutations were also detected in this NB cohort.

3.3 Rare Mutations

Mutations in FGFR-1, -2, and -3 were detected. The rare BRAF L588P mutation is close to the common V600E mutation in melanoma and was found in 5.17% of the

Table 2. List of clinical significant SNV mutations detected by NGS on DNA of somatic tumor tissues of Relapsed or Refractory NB patients.

Gene	%	SNV, clinical significant mutation	Relation with clinical properties
<i>ERBB2</i>	39.65	I655V	No, $p > 0.05$
<i>ALK</i>	29.31	F1174L, R1245Q , H1124R, G1125S, A1126T, V1135A, L1152R, I1171T, S1189F, E1197K, D1203G, T1211P, R1214C, P1215L, V1229L, E1241G, H1244R, F1271L	No, $p > 0.05$
<i>ABL1</i>	27.59	M244V	No, $p > 0.05$
<i>ATM</i>	27.59	D1853N, D1853V, F582L, L615P, P872S	No, $p > 0.05$
<i>KIT</i>	22.41	M541L, N486T E583L M552T, I438V, W557R, L576P, V824A,	No, $p > 0.05$
<i>P53</i>	22.41	D281G, R175H, M246V, G245D, L194F, T163C, R209, P152L, R337H, R267G, P223S	No, $p > 0.05$
<i>RBI</i>	22.41	P793S , R661W, R441L, T356I,	No, $p > 0.05$
<i>EGFR</i>	18.97	Q97G, G598R, L745R, Q746L, V769M, N771D, R776H (DR), T790M, R831H, V843I	No, $p > 0.05$
<i>FGFR3</i>	18.97	L650E	No, $p > 0.05$
<i>FGFR2</i>	17.24	D374G	No, $p > 0.05$
<i>APC</i>	13.79	P870S , G1116D	No, $p > 0.05$
<i>HNF1A</i>	13.79	R263H, frameshift,	No, $p > 0.05$
<i>FGFR1</i>	10.34	D133dup, R22S	No, $p > 0.05$
<i>JAK3</i>	10.34	S493G	No, $p > 0.05$
<i>CSFR1</i>	10.34	Q955D	No, $p > 0.05$
<i>MET</i>	10.34	N375S, S812P, N1117G, Y1248H,	No, $p > 0.05$
<i>VHL</i>	8.62	Q70G, S72P, L128P, V170A	No, $p > 0.05$
<i>PIK3CA</i>	6.89	F50L, R88G, R108H, L111R	No, $p > 0.05$
<i>NOTCH1</i>	5.17	R2455A	NA
<i>BRAF</i>	5.17	L588P, E451K, R444G, V459A, T470A	NA
<i>MAP2K</i>	5.17	D66G, F53L	NA
<i>HRAS</i>	5.17	G13V	NA
<i>KRAS</i>	5.17	G22R	NA
<i>FBXW7</i>	5.17	R658G	NA
<i>CDH1</i>	3.45	T340M	NA
<i>FLT3</i>	3.45	M837T	NA
<i>CTNNB1</i>	3.45	S33P, T41A	NA
<i>DDR</i>	3.45	S768G	NA
<i>NF1</i>	3.45	Q1841H, H1842P	NA
<i>MLH1</i>	1.7	V378P	NA
<i>STK11</i>	1.7	P280S	NA
<i>GNAS</i>	1.7	R201H	NA
<i>IDH1</i>	1.7	R132C	NA
<i>SMAD4</i>	1.7	R361C	NA
<i>CDK2A</i>	1.7	G101W	NA

Mutations in bold are frequently observed. NA, Not Assesed.

present NB cohort. This mutation could be considered for targeted therapy. Other BRAF mutations detected near the P-loop (G466) were E451K, R444G, V459A, and T470A.

3.4 RNA Fusions

Fusions were detected in 19.64% of cases using RNA NGS (Table 3) and may provide an opportunity for targeted therapy. The most common fusions were detected in *NTRK3*, *ROS1*, and *RET*. *NTRK1* gene fusion was detected in one case, *FGFR3* fusion in two cases, and *ALK*

fusion in one case. No fusions were observed in *NRG1*, *PBX1*, *FGFR2*, *EGFR*, *NTRK2*, *RAF1*, *MET*, *PPARG*, *ERG*, *PIK3CA* or *TFE3*.

3.5 Association of Clinical Features with Genetic Alterations

Clinical and pathological features such as age, gender and risk classification were not associated with the presence of any specific mutation ($p > 0.05$). As expected, the patient risk category was significantly correlated with stage

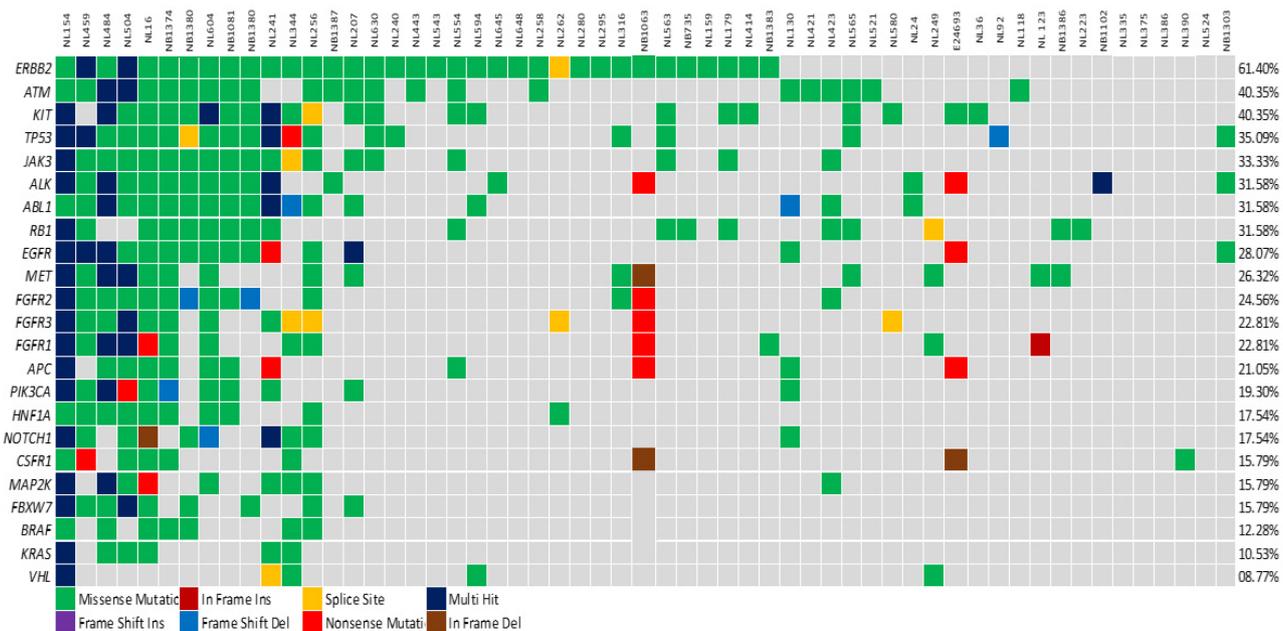


Fig. 1. Onco-plot of the cases according to frequency and type of mutations. 22.8% of the cases included more than 10 sense mutations.

Table 3. RNA fusions and partners among 18 genes and 87 fusions detected.

Gene	Fusion
<i>RET</i>	RET (e3):RET (e8) deletion
	RET (e11):KIF5B (e24)
<i>ROS1</i>	ROS1 (e35):CCDC6 (e1)
	ROS1 (e35):EZR (e10)
<i>NTRK</i>	NTRK1 (e9):TFG (e4)
	NTRK3 (e6):TFG (e4)
	NTRK3 (e6):EML4 (e14)
<i>ALK</i>	ALK (e20):EML4 (e14)
<i>FGFR</i>	FGFR3 (e17):TACC3 (e11)
<i>BRAF</i>	BRAF(e11):KIAA1549 (e15)

($p < 0.001$), N-Myc amplification ($p = 0.018$), NSE ($p = 0.007$), ferritin ($p = 0.019$) and LDH ($p = 0.021$) in Spearman correlation analysis.

In summary, predictive ALK mutations were detected in the tyrosine kinase domain. Five patients who received anti-ALK therapy showed regression or stable disease after a mean follow-up of 17 months. Five-year survival rates for all patients who received anti-ALK therapy will be reported in a future study.

A high incidence of the ERBB2 I655V mutation was found, but there is insufficient evidence to justify the use of trastuzumab deruxtecan for pediatric cancers. Mutations were also detected in *ABL-1*, *KIT*, *FGFR*, *PIK3CA*, *EGFR*, *MET* and *BRAF*. These mutations have predictive value in adult cancers, but their status in NB is not yet known. Our findings may be useful for refractory NB cases with

no other options. We also detected RNA fusions in *ROS1*, *RET*, *NTRK1*, *NTRK3*, *ALK*, and *BRAF* in 19.64% of cases. These fusions may be predictive for targeted therapy. The detection of *ATM*, *p53*, *RB1*, *APC*, *HNF1A*, *VHL*, *NOTCH*, *NF1*, and *RAS* mutations may be helpful for understanding the pathogenesis of NB.

4. Discussion

In the present study we report NGS mutation and fusion data for relapsed and refractory NB patients from our institute. We found several potential treatment targets, notably ALK mutations, fusions in *NTRK*, *ROS1* and *RET*, *BRAF* mutations other than V600E, the common ERBB2 I655V mutation, as well as other rare mutations for which the clinical relevance in NB has yet to be established.

The ERBB2 I655V missense mutation was common in this NB cohort. ERBB2 is located at 17q12 and is a well-known receptor tyrosine kinase involved in growth factor signaling. Clinical trials targeting ERBB2 are ongoing with agents such as fam-trastuzumab deruxtecan in non-small cell lung cancer and breast cancer. The only pathogenic mutation found in ERBB2 in our NB cohort was I655V. ERBB2 is altered in 5.44% of malignant solid tumors, including adenocarcinomas of the gastroesophageal junction (19.62%), urothelial carcinomas (14.87%), breast carcinomas (13.78%), gastric adenocarcinoma (10.08%), cervical carcinoma (9.09%), malignant uterine neoplasm (8.03%), colorectal carcinoma (4.69%), non-squamous non-small cell lung carcinoma (4.28%), non-small cell lung carcinoma (3.97%), biliary tract carcinoma (5.41%), glioblastoma (1.84%), WHO grade III glioma (1.55%), and some

diffuse midline glioma, H3 K27M-mutant patients. ERBB2 I655V mutation is an inclusion criterion in open clinical trials (mainly phase 2) for malignant solid tumors [37].

EGFR signaling is important for cell growth and proliferation in high-risk NB, and copy number gains that span ERBB2 are common. High levels of ERBB2 expression or amplification are used routinely to select HER2-positive breast cancer patients for targeted therapy with trastuzumab. ERBB2 is reported to be altered in 2.4% of NB patients and to date there has been only one clinical trial. ERBB2 I654 and I655V mutations are genetic risk factors for cancer [37].

Most NB cases with ALK mutations have N-Myc amplification [38]. Half the cases with ALK mutations in the present series also had N-Myc amplification. One of the observations in the present study that should be confirmed in a larger series is that familial cases with germline R1275Q mutations did not show N-Myc amplification.

Mutations in the FGFR pathway are observed in 7% of all cancers. Small molecule drugs are available that target FGFR, including pemigatinib, infigratinib, erdafitinib, derazantinib and futibatinib. Ongoing clinical trials in cholangiocarcinoma and metastatic urothelial carcinoma have shown encouraging results for FGFR inhibitors, either alone or in combination with immunotherapy. FGFR2 fusions or rearrangements, or loss of function mutations in the FGFR2 gene (FGFR2 D530N and FGFR2 A648T) could therefore be used for targeted therapy. In the present NB cohort we found FGFR3 (e17):TACC3 (e11) fusion and one case with FGFR1 D133 duplication. Disruption of FGFR activity could also change the NB microenvironment in terms of tumor-associated fibroblasts or macrophages, as well as immune cells such as T lymphocytes [39,40].

In addition to guiding targeted therapy, NGS results can be used for diagnosis and prognosis in various cancer types. The sequencing panel used in the present study does not provide much information for diagnosis, but the prognostic value of detected mutations should be explored in larger patient series. Anti-FGFR targeted therapies for NB should also be explored with regard to possible side-effects and tumor resistance.

The BRAF proto-oncogene is involved in the mitogen-activated protein kinase (MAPK) signaling pathway. The BRAF V600E mutation is considered to be a class I mutation type and is commonly found in melanoma, colorectal cancer and multiple myeloma, but is very rare in NB. New types of mutations (class II and class III) have now been defined in cancer, including aberrant transcriptional gene regulation. Class II mutations include non-V600E mutations in the RAS binding domain, RAS wild type, and RAS independent kinase activated cases. Class III mutations include the kinase domain, RAS-dependent, and RAS mutant cases [41]. We speculate the rare BRAF mutations observed in NB tissue with one or more RAS mutations could be classified as Class III mutations. These mutations are located

near the P-loop (G466). The prognostic and therapeutic role of BRAF mutations that coexist with KRAS mutations in NB require further investigation, together with the importance of the BRAF L588P mutation in the kinase domain.

Despite the small number of cases in our patient cohort, we found similar mutations to those reported in other recent studies of relapsing, therapy-resistant NB. A limitation of the present work is that the 60-gene panel does not include some genes related to NB, including N-Myc. NGS cannot replace Fluorescence In Situ Hybridization (FISH) or PCR for the detection of Segmental Chromosomal Aberrations (SCA). For example, Kim *et al.* [42] recently compared NGS and FISH for SCA of 1p deletion, 11q deletion and 17q gain in 35 NB samples. They concluded that NGS was a sensitive complementary and alternative method to conventional FISH for detecting SCAs in NB [42]. However, the NGS method is not yet accepted as a gold standard for the detection of SCA in NB. We are currently comparing the FISH, real-time PCR, digital PCR, and NGS methods in our laboratory for routine testing of N-Myc amplification and 11q deletion. The cost of NGS increases when SCA is added to the panel. It is preferable to use 8 DNA control samples to compare losses or gains of sequenced genes.

In general, routine NGS analysis is critical for the clinical management of patients with solid tumors. The use of NGS for pediatric cancers is also gaining widespread use. An experienced team that includes pathologists familiar with molecular techniques, molecular biologists/oncologists, bioinformaticians, and clinical oncologists is necessary to obtain and correctly interpret NGS data. Appropriate preanalytical procedures including good tumor tissue selection and storage are also very important. One of the challenges is how to report a rare mutation detected by the bioinformatic analysis that is not linked to a clinically proven indication for targeted therapy. The multidisciplinary team should be aware of the function of the relevant gene and whether targeted drugs exist for its protein.

We believe the importance of this research is that genes related to targeted therapy, even more than the known and approved molecular changes, may offer new opportunities for treatment in relapsed/ refractory NB patients. For successful molecular evaluation it is important to use methods that work well with low quality and/or low quantity tumor tissues, including bone marrow tru-cut biopsies that have undergone decalcification. Whole exome sequencing generally requires larger amounts of sample DNA. Targeted somatic exome sequencing using CLIA-certified NGS platforms such as MSK-IMPACT or Foundation One NGS panels may provide other choices that avoid whole genome sequencing [43]. The whole exome comprises 1.5% of the entire genome, including the coding regions [44].

Samoyedny *et al.* [45] reported the adequacy of NGS was 88% in a study of 95 cases of NB-positive biopsies. Their series included image-guided biopsies for relapsed NB. NGS adequacy was also reported to be 82% for bone

tumor biopsies and 96% for soft tissue tumor biopsies [45]. In the present NB series, an NGS adequacy of 100% was achieved through a step-by-step evaluation of the preanalytical quality and quantity of sample. We prefer primary biopsy or surgical tissue material obtained prior to the use of neoadjuvant therapy. Starting with fresh frozen tissue or FFPE, we select blastic tumor areas without necrosis, hemorrhage, or normal tissue. If this is not available, we use lymph node, soft tissue, and bone marrow biopsies that contain metastatic tissue, and macrodissect the tumor blastic tissue with high purity into a microtube. If the quantity is low following DNA and RNA extraction, the extraction procedure is repeated from the beginning.

From the perspective of bench to bedside, the rare mutations detected in this study may lead to targeted therapy trials in relapsed and/or refractory NB patients. These mutations may offer clues for the development of new targeted drugs, or for improving existing targeted drugs so they can act on the known target as well as the rare loci. In future, we will continue NGS molecular evaluation of NB cases by improving the panel, in parallel with the International NB study group reports. We also plan to use NGS for all cases at diagnosis and to routinely test for N-Myc amplification, 11q24 deletion and possibly DNA ploidy, together with other panels related to targeted therapy. The future NGS plans for our Molecular Oncology Laboratory will consider the latest reports from the College of American Pathologists, the Association of Molecular Pathology, and the Catalog of Somatic Mutations of Cancer database [46,47].

For cases where tumor tissue is not available, we have also started an NGS liquid biopsy panel. We will examine liquid biopsy in a prospective study by detecting DNA in plasma for the follow-up of minimal residual disease in NB. Successful NGS using liquid biopsy has been reported for many cancer types including NB [48], although fewer selected genes are studied with this sample type. For example, a panel of 8 oncogenes (*EGFR*, *KRAS*, *BRAF*, *HER2*, *MET*, *ALK*, *RET*, and *ROS1*) was used to study non-small cell lung cancer [49]. Multicancer liquid biopsy panels are also sold commercially, but none are yet available for NB or pediatric cancers. Customized panels could be designed, or a commercial panel suitable for pediatric cancers could be used, such as the multi-cancer cfDNA panels for sequencing more than 30 genes. Ideally, a customized panel for cfDNA and designed for NB would include the mutations found in the present study and in the literature.

Pugh *et al.* [50] used whole exome sequencing to search for mutations in paired samples of NB tissue and peripheral blood. They found a low somatic mutation burden that included mutations in *ALK* (9.2%), *PTPN11* (2.9%), *ATRX* (2.5), *MYCN* (1.7%), and *NRAS* (0.83%). Their NB series was significantly enriched for pathogenic germline variants in *ALK*, *CHEK2*, *PINK1*, and *BARD1* [50]. In the present series we only searched for germline mutations

when familial NB was suspected clinically. This was a limitation of our study, but the main aim was to investigate mutations related to targeted therapy. Another limitation of this study was that important NB-associated mutations such as *ATRX* and *MYCN* may have been missed due to the targeted NGS of selected genes from a cancer panel. However, this ready-to-use panel results in far fewer NGS failures. The panel also includes genes for which targeted therapy drugs are already available.

5. Conclusions

In conclusion, the NB cases in this study showed mainly *ALK* F1174L (sensitive to alectinib), and R1275Q (sensitive to crizotinib) mutations. Rare mutations were also found in the tyrosine kinase domain. *EGFR*, *ERBB2*, *BRAF*, *ABL1*, *MET* and *PIK3CA* mutations were also found in NB, but their clinical importance remains to be determined. This study supports the detection by NGS of SNV mutations as a useful tool to identify predictive, diagnostic and prognostic markers in NB. The use of methods and validated kits that are suitable for individual molecular oncology laboratories is necessary. Appropriate panel selection is also important. An important challenge is to obtain good results with low quality and low-input tissues. Although infrequent, fusions should also be routinely investigated in NB patients to identify additional opportunities for targeted therapy.

Availability of Data and Materials

The data might be shared upon request anonymously after ethics approval.

Author Contributions

TA, SA, NO designed the research study. DK, DI, EC, NO, EO collected data and patients. TA, SA, ES, AE, OG, SMO, ZA performed the research and laboratory work. TA, SA analysed the data. TA, SA, OG, NO wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

Ethical Approval was taken from Dokuz Eylul University Non-invasive Research Ethics Committee (14.12.2022, No: 2022/40-03). Informed Consent was signed to parents of the patients.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/j.fbl2808171>.

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