

Genetic Alterations Detected by Targeted Next-generation Sequencing and Their Clinical Implications in Neuroblastoma

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Abstract. *Background/Aim:* This study was conducted to evaluate the clinical usefulness of panel next-generation sequencing (NGS) and to investigate the spectrum of genetic alterations and their clinical implications in neuroblastoma. *Patients and Methods:* Formalin-fixed, paraffin-embedded archival samples from 41 cases of neuroblastoma were used for targeted sequencing. *Results:* A total of 145 somatic mutations were identified, including 51 synonymous, 86 missense, 3 nonsense, 2 frameshift deletion, 2 splice-site, and 1 in-frame deletion mutations. The most frequently mutated gene was ALK (9 missense mutations). The common copy number variations (CNVs) were amplification at 2p24.2 and deletion at 11q22.3 and 1p36.21. ALK mutations were more frequent in patients with stage 4 or 4S (0% vs. 33.3%, $p=0.017$). Among 27 patients with high-risk disease, the 5-

year overall survival was inferior in patients with ALK mutations to those without (25.0% vs. 67.0%, $p=0.009$). *Conclusion:* Genetic analysis using targeted NGS was feasible and helpful in detecting point mutations and CNVs in neuroblastoma. Targeted NGS could predict prognosis and be used to find molecular target-based therapies for neuroblastoma.

Neuroblastoma is the most common extracranial pediatric solid tumor, accounting for 8-10% of all childhood malignancies (1). Its behavior is very diverse, with some tumors regressing spontaneously, while other high-risk tumors show very aggressive features that frequently lead to relapse. This necessitates a multimodal treatment approach including surgery, chemotherapy, high-dose chemotherapy with autologous stem cell transplant, immunotherapy, and radiotherapy. Despite this aggressive multimodal approach, clinical responses vary widely, with over 90% cure rates in patients with low-risk disease and 50 to 60% cure rates for those with high-risk disease (2-6).

Genetic abnormalities, including point mutations in genes such as ALK or copy number variations (CNVs) such as MYCN amplification, have been reported to have prognostic value for neuroblastoma (7). Genomic assay using next-generation sequencing (NGS) can reveal multiple genetic alterations in neuroblastoma in one run. Clinical NGS testing mainly focuses on the detection of mutations such as single-nucleotide polymorphisms (SNPs) and small insertion and deletion (indel) mutations. In addition, CNVs can also be detected by targeted NGS using a software package developed for this purpose.

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The recent introduction of oncogene targeted sequencing has revolutionized the treatment scheme for many cancers. Detecting tumor-associated genetic aberrations makes it easier to predict outcomes and to find targeted therapies. However, studies on the usefulness of NGS panels for pediatric cancer are rare, and few studies have evaluated the feasibility and usefulness of targeted panel sequencing for neuroblastoma (8, 9). This study evaluated the clinical usefulness of NGS panels in detecting SNPs and CNVs in archived formalin-fixed paraffin-embedded (FFPE) neuroblastoma samples and investigated the spectrum of genetic alterations and their clinical implications in neuroblastoma.

Patients and Methods

Patients and tissue samples. We retrospectively reviewed medical records of 41 patients diagnosed with neuroblastoma between Jan 2002 and Dec 2015s at the Asan Medical Center, Seoul, Korea who had archived formalin-fixed paraffin-embedded (FFPE) samples. The institutional review board of Asan Medical Center approved the study.

FFPE archival samples were used for targeted NGS. Two pathologists reviewed the samples. The tumor area was marked to determine tumor purity and to guide tumor DNA extraction.

DNA extraction. Depending on the sample size and tumor cellularity, genomic DNA was extracted from two to five 6- μ m-thick slices per FFPE tissue sample. After de-paraffinization with xylene and ethanol, genomic DNA was purified using a NEXprep FFPE Tissue kit (#NexK-9000; Geneslabs, Seongnam, Republic of Korea) according to the manufacturer's recommendations. Briefly, tissue pellets were lysed completely by incubation with proteinase K in lysis buffer overnight at 56°C, followed by an additional incubation for 3 min with magnetic beads and Solution A at room temperature. After incubation for 5 min on a magnetic stand, the supernatants were removed, and the remaining beads were washed three times with ethanol. After air-drying the beads for 5 min, DNA was eluted in 50 μ l nuclease-free water and quantified using a Qubit™ dsDNA HS Assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

Targeted NGS. To evaluate genetic alterations, targeted NGS was performed with OncoPanel AMC version 1 (OP_AMCv1, developed in-house by ASAN Center for Cancer Genome Discovery targeting a total of 176 genes, including the entire exons of 164 genes, the partial introns of 39 genes that are often rearranged in solid cancer, and additional small-sized (10,034 bp) specific SNP loci for genetic fingerprinting. With the MiSeq platform (Illumina, San Diego, CA, USA), a DNA library was constructed as described in our previous report (10). Briefly, gDNA shearing with covaris sonication, end repair, A-tailing, and ligation was performed with a TruSeq adaptor using the SureSelect XT reagent kit (Agilent Technologies, Santa Clara, CA, USA). Each library was addressed with sample-specific barcodes, 6 bp in size, and quantified using the Qubit kit. Eight libraries were pooled (yielding a total of 720 ng) for hybrid capture using the Agilent SureSelect XT custom kit (OP-AMCv1 RNA bait, 1.17 Mb; Agilent Technologies). The concentration of the enriched target was measured using quantitative polymerase chain reaction (qPCR; Kapa Biosystems, Inc., Woburn, MA, USA), and then, DNA

libraries that passed quality checks were loaded onto the MiSeq platform for paired-end sequencing.

Bioinformatics analysis. Sequenced reads were aligned to the human reference genome (NCBI build 37) with the Burrows-Wheeler Aligner (0.7.17) using default options. De-multiplexing was performed with MarkDuplicates of the GATK4 package to remove PCR duplicates (11). De-duplicated reads were re-aligned at known indel positions with the GATK4 BaseRecalibrator tool (version 4.1.3.0), base quality was recalibrated using the GATK4 ApplyBQSR tool, and somatic variant calling for single nucleotide variants and short indels was performed with the Mutect2 tool. Germline variants from the somatic variant candidates were filtered out using the Single Nucleotide Polymorphism database (dbSNP, build 141; found in >1% of samples), Exome Aggregation Consortium database (ExAC; r0.3.1, threshold frequency 0.001), Korean Reference Genome database (KRGDB), and an in-house panel of normal controls. After additional filtering using GATK4 FilterMutectCalls tools, final somatic variants were annotated using the Variant Effect Predictor (version 86), and were then converted to the Mutation Annotation Format (MAF) file format using vcf2maf (12). False-positive variants were manually curated using the Integrative Genomics Viewer (IGV). For the analysis of structural variations, CNVs and rearrangements were evaluated using the CNVkit (0.9.6), GISTIC (2.0.23), and BreakMer (0.0.6) algorithms, respectively. After analysis of the CNVs, the GISTIC algorithm was applied to the segmented files (CNS) from the CNVkit output for the identification of significant focal and arm level amplifications and deletions. The GISTIC value cut-off was set at 0.25 per the software's instructions. Candidates for germline mutations or false positives for rearrangement alterations by BreakMer were filtered out with an in-house panel of normals and by manual review.

Statistics. Categorical variables were assessed using Fisher's exact test. Relapse-free survival (RFS) was calculated from the date of diagnosis to the date of relapse. Overall survival (OS) was calculated from the date of diagnosis until death, or until data were censored. The Kaplan-Meier method was used to produce OS and RFS rates. All *p*-values <0.05 were considered statistically significant. All statistical analyses were performed using SPSS version 21.0 (IBM Inc., Armonk, NY, USA).

Results

Patient characteristics. Table I shows the clinical characteristics of patients with neuroblastoma included in this study. The median age at diagnosis was 25 months (range=0-120 months). Sixteen patients (39%) were less than 18 months old at diagnosis. Nineteen patients (46%) were males. The primary site was the adrenal gland in 26 patients (63%). Four patients had stage 1, two patients had stage 2, six patients had stage 3, twenty-four patients had stage 4, and three patients had stage 4S disease. According to the Children's Oncology Group (COG) risk stratification (13), 10 patients (24%) had low-risk, 4 (9.7%) had intermediate-risk, and 27 (66%) had high-risk disease. Of the 27 patients with high-risk disease, 17 experienced relapses, and 15 of them died of progressive disease. In addition, one patient died of

Table I. Patient characteristics and outcomes.

Characteristics	Number
Age at diagnosis, n (%)	
Median, year (range)	2.3 (0-10.0)
<18 months	16 (39)
≥18 months	25 (61)
Gender, n (%)	
Male	19 (46)
Female	22 (54)
Primary site, n (%)	
Adrenal gland	26 (63)
Others	15 (37)
INSS stage, n (%)	
1	4 (10)
2	4 (10)
3	6 (15)
4	24 (59)
4s	3 (7)
Risk stratification, n (%)	
High	27 (66)
Intermediate	4 (10)
Low	10 (24)
Relapse or progression, n (%)	
Yes	18 (44)
No	23 (56)
Survival status, n (%)	
Alive	25 (61)
Dead	16 (39)

INSS: International Neuroblastoma Staging System.

treatment-related causes without disease progression. Overall, 11 of 27 high-risk patients were alive without disease at the last follow-up. Thirteen of the 14 patients with low-risk or intermediate-risk disease survived and were disease-free at the last follow-up, and one patient experienced relapse but was alive without disease at the time of the last follow-up.

Significantly mutated genes in neuroblastoma. The mutation profiles are summarized in Figure 1. A total of 145 somatic mutations were identified in the protein-coding region, including 51 synonymous mutations, 86 missense mutations, 3 nonsense mutations, 2 frameshift deletion mutations, 2 splice-site mutations, and 1 in-frame deletion mutation. The median number of somatic mutations in the protein-coding region per sample was 2 (range=0-12) with an average of 6.3 mutations/Mb (range=0-25.2). SNV class was predominantly C > T or T > C transitions (Figure 1A).

The functional mutations in the top 20 most frequently mutated genes are summarized in Figure 1B. The most frequently mutated gene was *ALK* (n=9; missense mutations in 9). Other recurrently mutated genes were *NFI* (n=4; missense mutation in 2, frameshift deletion in 1, and splice site mutation in 1), *NOTCH4* (n=3; missense mutations in 3),

FLT1 (n=3; missense mutations in 3), *BRCA2* (n=3; missense mutations in 3), *ERBB3* (n=2; missense mutations in 2), *ERBB2* (n=2; missense mutation in 1 and in-frame deletion in 1), *ATM* (n=2; missense mutation in 1 and nonsense mutation in 1), *APC* (n=2; missense mutations in 2), and *ABL2* (n=2; missense mutations in 2). Figure 1C shows a VAF plot of the mutated genes; mutations in *NOTCH4*, *ATM*, *BRCA2*, *ERBB3*, *MSH2*, and *KDR* may be germ-line mutations that were not filtered by the analysis pipeline.

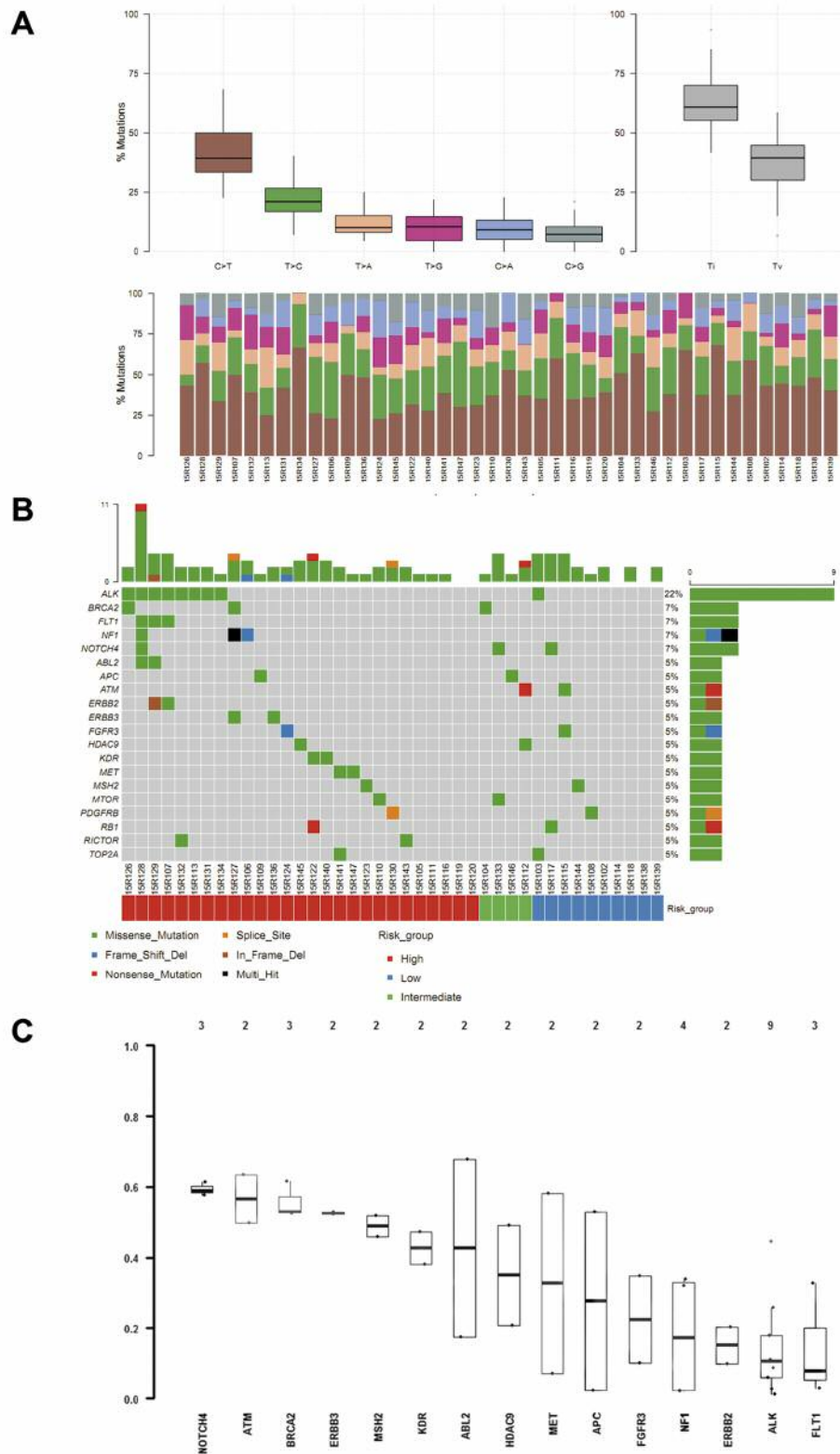
The locations of the mutations in the *ALK* gene are summarized in Figure 2. Eight *ALK* mutation spots occurred in the kinase domain (I1171T in 1, I1171N in 1, F1245C in 2, and R1275Q in 4), and one occurred in the MAM domain (R292H). All spots in the kinase domain were previously reported as pathogenic mutations in the ClinVar database.

NOTCH4 mutations were found in 1 patient with low-risk disease, 1 patient with intermediate-risk disease, and 1 patient with high-risk disease. *FLT1* mutations were found in 3 patients with high-risk disease, and all of these patients also had *ALK* mutations. *BRCA2* mutations were found in 2 patients with high-risk disease and 1 patient with intermediate disease.

Copy number variations. A GISTIC2 analysis was additionally performed to detect focal CNVs. The most common focal amplified region was located at 2p24.2 (12/41, 29%), which contains *ALK*. The most common focal deleted region was at 11q22.3 (14/41, 34%), followed by 1p36.21 (11/41, 27%) (Figure 3).

Clinical characteristics and outcomes according to genetic alterations. *ALK* mutations were more frequent in patients with stage 4 or 4S disease (9/27, 33.3%) than in patients without distant metastases (0/14) ($p=0.017$). *ALK* mutations were more frequent in patients with high-risk disease (8/27, 29.6%) than in patients with intermediate or low-risk patients (1/15, 7.1%), but this difference was not statistically significant ($p=0.131$). The *ALK* mutation frequency did not differ according to the primary site or age at diagnosis. Among 27 patients with high-risk disease, the 5-year RFS and OS of patients with *ALK* mutations was inferior to those without mutations (RFS: 15.0% vs. 47.4%, $p=0.038$, OS: 25.0% vs. 67.0%, $p=0.009$) (Figure 4A and B).

The frequency of amplification of 2p24.2 and deletion of 11q22.3 was not significantly different according to the risk group, while deletion of 1p36.21 was exclusively found in the high-risk group ($p=0.007$). Amplification of 2p24.2 and deletion of 11q22.3 did not have an impact on survival outcomes, while deletion of 1p36.21 was significantly associated with inferior 5-year RFS and OS among high-risk patients (RFS: 27.3% vs. 46.7%, $p=0.016$; OS: 27.3% vs. 73.9%, $p=0.003$) (Figure 4C and D).



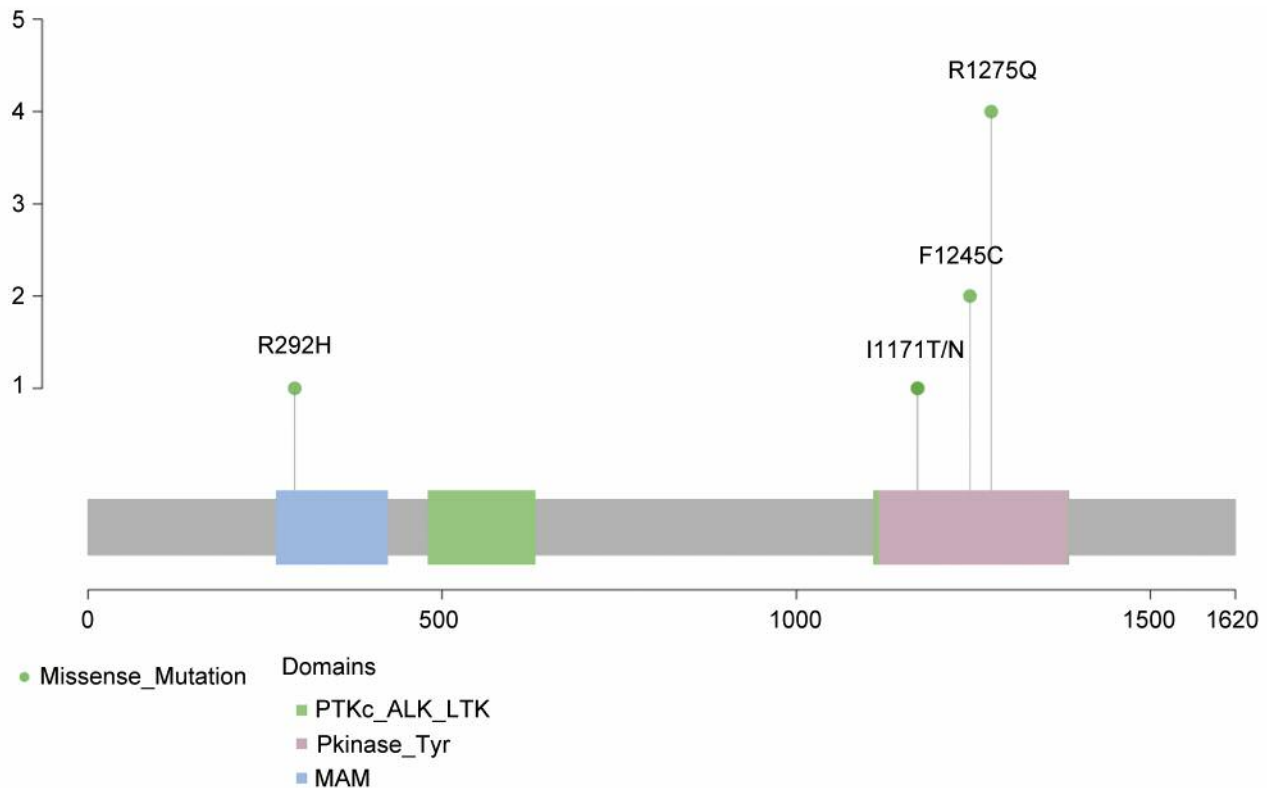


Figure 2. Lollipop plots of the identified somatic mutations in ALK.

Discussion

In this study, we performed a targeted NGS using archived FFPE neuroblastoma samples to evaluate the frequency of genetic mutations in various risk groups and their clinical implications. Our study showed that this approach was feasible and easily applicable, and that it can help detect clinically significant somatic mutations in cancer-related genes in neuroblastoma. The results showed that *ALK* mutations were the most common somatic aberrations, and were also associated with clinical outcomes. Also, focal deletion of 11q22.3 and 1p36.21 was commonly detected.

Previous studies have reported that somatic mutations in sporadic neuroblastoma included mutations in *ALK*, *ARID1A*, *ARID1B*, and *ATRX* (14-19). Each of these mutations has been reported to be associated with specific clinical features and outcomes (20). Among them, mutations in *ALK* are one of the most common alterations. Previous studies have reported that activating mutations in *ALK* occurred in 7 to 8% of sporadic neuroblastoma cases (14, 15, 21). PeCan Cloud reported that *ALK* mutations were the most common somatic mutations in neuroblastoma, occurring in 41 out of 495 patients (8.2%) (22). We found that 9 out of 41 total patients (22.0%) and 8 out of 27 high-risk patients (29.6%) harbored

ALK mutations in their tumors. This frequency is somewhat higher than what has been reported in previous studies (14, 15, 21). The high prevalence of *ALK* mutations might be associated with the higher percentage of high-risk patients in our cohort compared to other studies, or the technique used, since, in a deep sequencing study of primary neuroblastoma, *ALK* F1174 and R1275 mutations were identified in 10% (27/277) of patients; half of these mutations were not detected by Sanger sequencing (23). The frequency of *ALK* mutations might also be skewed due to the relatively small number of patients in our study.

Our findings suggest that aberrations in RTK-RAS signaling pathways play a role in the tumorigenesis of neuroblastoma, and the presence of these aberrations may identify a high-risk population. The *ALK* gene belongs to the RTK superfamily. The clinical implications of *ALK* mutations in neuroblastoma have been reported to have various significance. In a meta-analysis, *ALK* mutations were found in 6.9% of 709 investigated tumors, and mutations were found in similar frequencies in neuroblastomas with favorable and unfavorable outcomes ($p=0.087$) (21). In contrast, Weiser *et al.* found a statistically significantly higher number of *ALK* mutations in patients with high-risk neuroblastoma when compared with low-risk cases

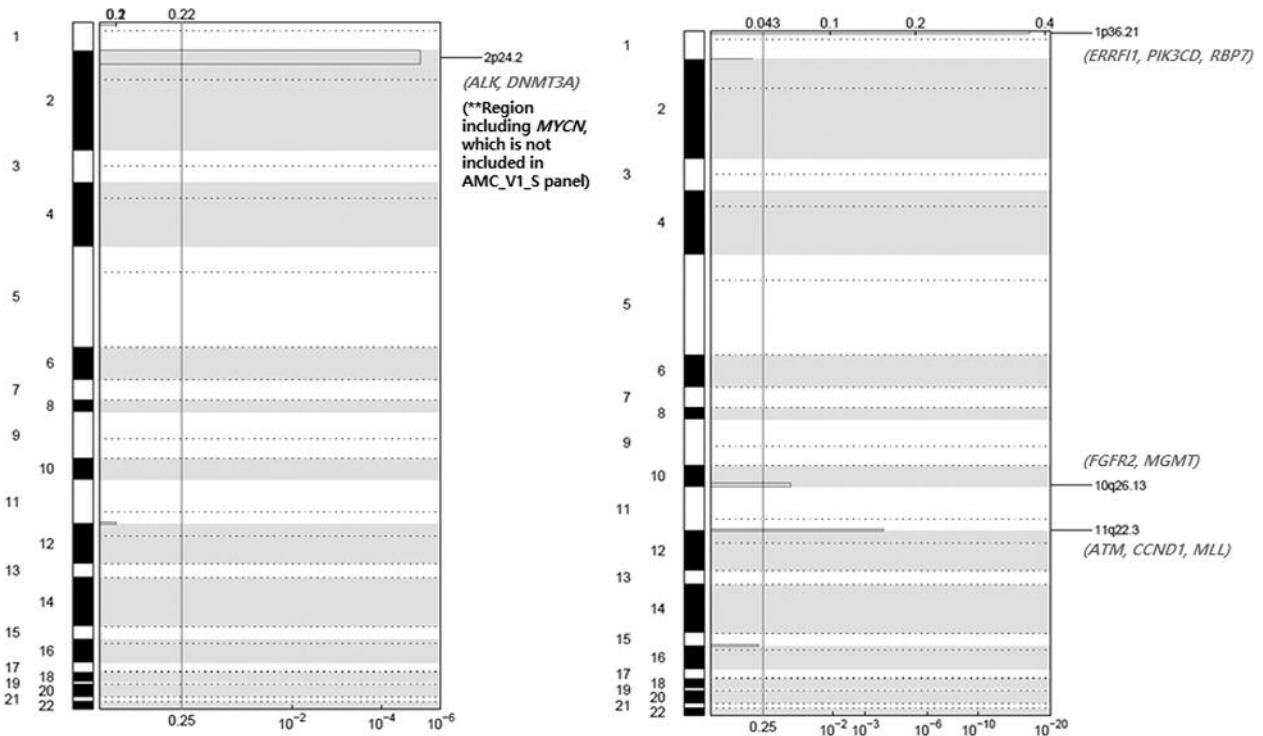


Figure 3. Copy number variations (CNVs) in neuroblastoma. Somatic CNV data for focal amplifications and deletions were analyzed using GISTIC2.0. The genome is oriented vertically from top to bottom, and the GISTIC q-values at each locus are plotted on a log scale from left to right. The vertical line represents the significance threshold ($q\text{-value}=0.25$). DEL: Deletion; AMP: amplification.

($p=0.018$) and cases with *MYCN* amplification ($p=0.031$) (24). In another study, 12 of the 13 mutations were found in patients with advanced stage neuroblastoma, regardless of *MYCN* amplification status (14). In another study, *ALK* mutations were found in 7 out of 93 samples (8%), and most of the patients with somatic *ALK* mutations had metastatic disease, characterized by *MYCN* amplification (15). In our study, the frequency of *ALK* mutations was higher in high-risk patients than in low/intermediate-risk patients, but this difference was not statistically significant (29.6% vs. 7.1%, $p=0.131$). In addition, the frequency of *ALK* mutations was significantly higher in patients with stage 4 or 4s disease than in patients without distant metastasis. This finding suggests that the presence of *ALK* mutations might be associated with a tendency for neuroblastoma to metastasize.

In the present study, *ALK* mutations were identified in the kinase domain, and R1275 was the most commonly aberrated locus, followed by F1245. Three hotspot residues (F1174, F1245, and R1275) have been reported to account for 85% of the *ALK* mutations seen in neuroblastoma (25). Identification of the *ALK* mutations is important because somatic mutations of the *ALK* receptor in neuroblastoma are the first therapeutically targetable genetic aberrations, and only targetable aberrations have been identified so far in this disease (25, 26). In addition,

the precise spot should be further investigated as a potential target for *ALK* inhibitors, because F1174L, which occurs frequently in neuroblastoma, only partially responds to crizotinib or alectinib, but responds to the third-generation *ALK* inhibitor lorlatinib (25). F1174L is considered to be the most aggressive of all *ALK* mutations. Due to the small number of patients, we were not able to assess the clinical significance of mutations at each locus. Still, in the present study, patients with *ALK* mutations showed universally poor OS compared to those without *ALK* mutations even in the high-risk group (5-year OS: 25% vs. 67%).

Less frequent recurrent mutations were found in *FLT1*, *NOTCH4*, and *BRCA2* genes. The clinical significance of these genes has not been revealed for neuroblastoma. *FLT1* is a cognate receptor of VEGF, and its activation can lead to activation of MAPK/ERK1/2 and subsequent neuroblastoma survival (27). Notch pathway signaling plays a critical role in differentiation, proliferation, and survival, and is oncogenic or tumor-suppressing in a variety of malignant tumors. It has been reported that activation of the NOTCH pathway leads to growth arrest in neuroblastoma cells (28). Germline *BRCA2* mutations have been reported to be detected in neuroblastoma patients. In these cases, parental genetic counseling and medical action is recommended (29).

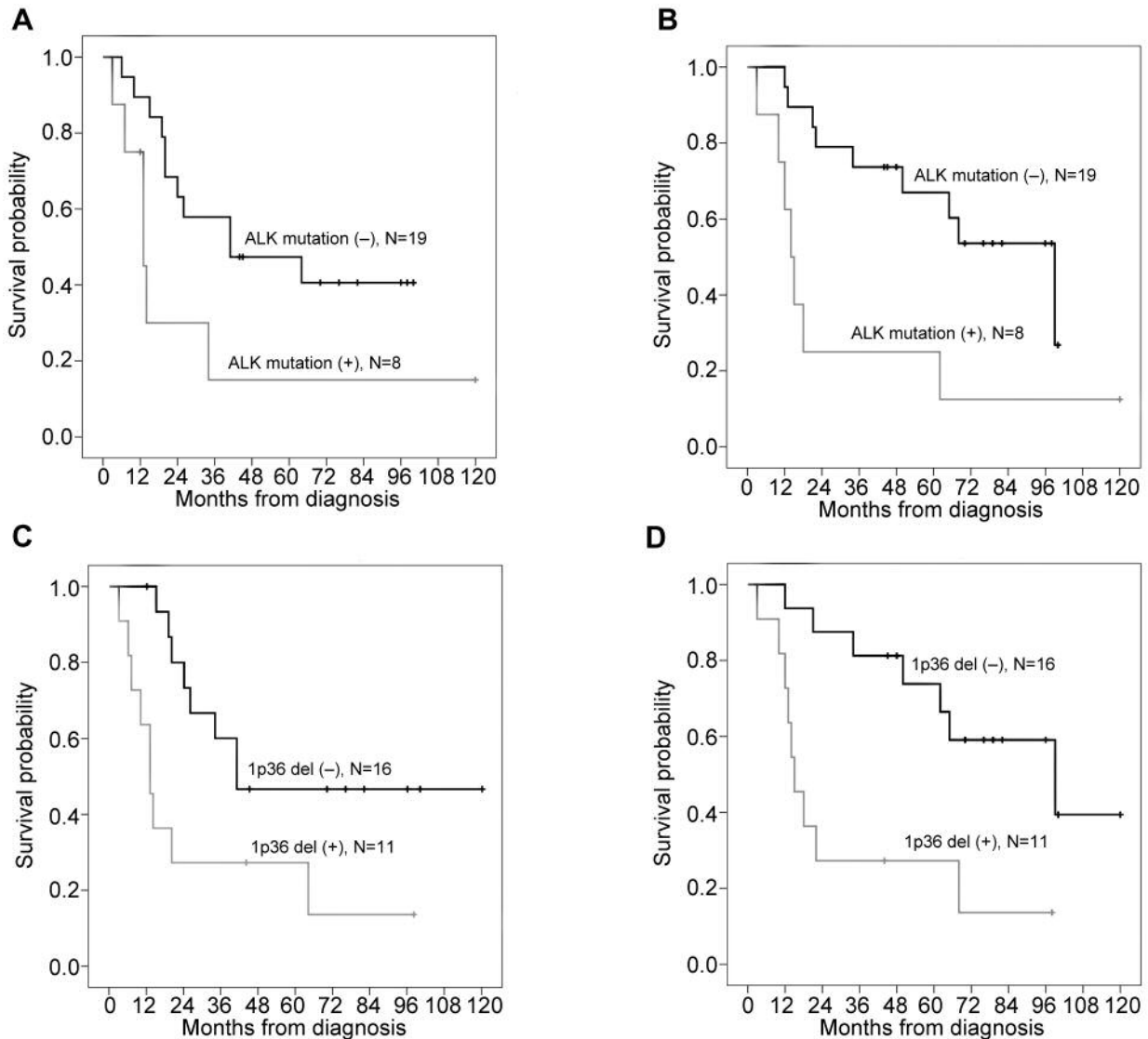


Figure 4. The 5-year relapse-free survival (RFS) (A) and overall survival (OS) (B) of 27 high-risk neuroblastoma patients with and without *ALK* mutations (RFS: 15.0% vs. 47.4%, $p=0.038$; OS: 25.0% vs. 67.0%, $p=0.009$), and the 5-year RFS (C) and OS (D) of 27 high-risk neuroblastoma patients with and without 1p36.21 deletion (RFS: 27.3% vs. 46.7%, $p=0.016$; OS: 27.3% vs. 73.9%, $p=0.003$).

Somatic mutations in the process of tumorigenesis generate unique mutational signatures detectable in individual tumors. A previous study suggested, that analysis of mutational signatures could identify high-risk patients with neuroblastoma (30), though we found no clinical implications for mutational signatures in our cohort. Further investigation is necessary to understand the clinical significance of mutational signatures in neuroblastoma.

We also analyzed somatic CNVs and found focal deletions and amplifications with significance in the GISTIC2 analysis. Focal deletion of 11q22.3 was commonly detected in tumors in the present study. Unbalanced deletion of 11q

is associated with inferior RFS in patients with otherwise low or intermediate risk (31). However, we did not find any prognostic associations related to 11q23 loss due to the small number of patients. *ALK* amplification was observed in 12 out of 41 patients (29.3%), which is higher than that observed in previous reports that found an *ALK* amplification in about 2-3% of patients, and in up to 15% of *MYCN* amplification cases (21). Since our study utilized the GISTIC pipeline, CNV detection might be overestimated. *ALK* amplification had no independent prognostic value in the present study, and this result is consistent with previous studies (21). The 1p loss of heterozygosity is known to

define a very aggressive subset of neuroblastoma, especially in the presence of *MYCN* amplification (1). Consistent with previous reports, 1p deletion was found only in high-risk patients, and associated with poor outcomes in our study.

Our study has several limitations. Because the NGS panel used in the present study was not designed specifically for use in pediatric cancer or neuroblastoma, *ATRX*, *ARID1*, *ARID2*, and *MYCN* were not included in the NGS panel. In addition, the study was a retrospective study, with a small number of patients. However, our study aimed to evaluate the feasibility of targeted NGS in neuroblastoma and showed that targeted NGS could be easily applied using archived FFPE samples, and can detect drug targets such as *ALK* mutations. This suggests that a more specific panel for neuroblastoma could yield more productive results and provide clinical utility.

In conclusion, genetic analysis using targeted NGS is feasible and helpful in detecting point mutations and CNVs, thus helping to predict prognosis of neuroblastoma. Our study revealed that a significant portion of high-risk patients had *ALK* mutations and a poor prognosis. The clinical benefit of NGS will be clearer if the efficacy of targeted agents such as *ALK* inhibitors is evaluated. Further studies that use targeted NGS for neuroblastoma and studies that evaluate molecular target-based therapies will be warranted to improve outcomes and decrease the toxicity of intensive conventional treatments for neuroblastoma.

Conflicts of Interest

The Authors declare that they have no conflicts of interest in relation to this study.

Authors' Contributions

Kyung-Nam Koh: Conceptualization, data curation, formal analysis, writing the original draft, and writing, reviewing and editing, Funding acquisition. Ji-Young Lee: Methodology, formal analysis, and writing, reviewing and editing. Jinyeong Lim: Methodology, formal analysis, and writing-review and editing. Juhee Shin: Data curation and writing, reviewing and editing. Sung Han Kang: Data curation and writing, reviewing and editing. Jin Kyung Suh: Data curation and writing-review and editing. Hyery Kim: Investigation and writing, reviewing and editing. Ho Joon Im: Supervision, writing, reviewing and editing. Jung-Man Namgoong: Resources and data curation and writing, reviewing and editing. Dae Yeon Kim: Resources and supervision. Se Jin Jang: Resources, supervision and writing, reviewing and editing. Sung-Min Chun: Conceptualization, methodology, data curation, formal analysis, writing the original draft, and writing, reviewing and editing.

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