Discovery of actionable genetic alterations with targeted panel sequencing in children with relapsed or refractory solid tumors

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Abstract

Advances in genomic technologies and the development of targeted therapeutics are making the use of precision medicine increasingly possible. In this study, we explored whether precision medicine can be applied for the management of refractory/relapsed pediatric solid tumors by discovering actionable alterations using targeted panel sequencing. Samples of refractory/relapsed pediatric solid tumors were tested using a targeted sequencing panel covering the exonic DNA sequences of 381 cancer genes and introns across 22 genes to detect clinically significant genomic aberrations in tumors. The molecular targets were tiered from 1 to 5 based on the presence of actionable genetic alterations, strength of supporting evidence, and drug availability in the Republic of Korea. From January 2016 to October 2018, 55 patients were enrolled. The median time from tissue acquisition to drug selection was 29 d (range 14–39), and tumor profiling was successful in 53 (96.4%) patients. A total of 27 actionable alterations in tiers 1–4 were detected in 20 patients (36.4%), and the majority of actionable alterations were copy number variations. The tiers of molecular alterations were tier 1 (clinical evidence) in 4 variants, tier 2 (preclinical evidence) in 8 variants, tier 3 (consensus opinion) in 2 variants, and tier 4 (actionable variants with a drug that is available in other countries but not in the Republic of Korea) in 9 variants. In one patient with relapsed neuroblastoma with \( \text{ALK} \) F1174L mutation and \( \text{ALK} \) amplification, lorlatinib was used in a compassionate use program, and it showed some efficacy. In conclusion, using a targeted sequencing panel to discover actionable alterations in relapsed/refractory pediatric solid tumors was practical and feasible.
**Introduction**

The outcome of pediatric cancer has improved substantially over the past few decades; however, the prognosis of relapsed/refractory pediatric cancer remains poor, and a new approach is needed to improve the outcome. Recently, the tremendous progress in molecular biology has enhanced our understanding of tumorigenesis and cancer cell survival at the molecular level [1]. These advances have ultimately led to the development of targeted therapeutics, which directly inhibit the pathways responsible for tumorigenesis. Biomarker-driven targeted therapy has already been successfully implemented in clinical practice in adult oncology, such as in use of an epidermal growth factor receptor inhibitor to treat non-small cell lung cancer [2].

Recent advances in genomic technologies have improved the ability to detect diverse somatic and germline genomic aberrations in cancer patients. Together, the advances in genomic technology and targeted therapeutics are increasingly making possible the development of precision medicine, which can be applied regardless of cancer pathology. Several recent studies exploring the feasibility of a precision cancer medicine approach in pediatric oncology have been published [3–8], and these demonstrated that the application of clinical genomics was possible and that a substantial number of patients had actionable genetic alterations, indicating the potential for targeted therapy.

In this study, we explored the possibility of applying precision medicine to the management of refractory/relapsed pediatric solid tumors by discovering actionable alterations using targeted panel sequencing.

**Materials and methods**

**Patients and sample collection**

Patients with relapsed or refractory solid tumors, who were younger than 18 years at initial diagnosis, were considered eligible. Samples taken at the time of relapse were preferentially used for genomic analysis; however, stored samples taken at the time of diagnosis were used when samples at relapse could not be obtained. Both fresh frozen (FF) tissue and formalin-fixed, paraffin-embedded (FFPE) tissue samples were used. All tumor specimens were examined by a pathologist to determine the percentage of viable tumor cells in each sample and adequacy for sequencing.

This study was approved by the Institutional Review Board of Samsung Medical Center (IRB approval no. SMC 2015-11-053), and written informed consent was obtained from the participants and/or their parents or legal guardians.

**Targeted sequencing**

A targeted sequencing panel (CancerSCAN™) to cover the exonic DNA sequences of 381 cancer genes and introns across 22 genes for rearrangement detection was used (S1 Table). This panel originally designed by the Samsung Genome Institute is available through company GENINUS. Tumor DNA (200 ng for FF or 300 ng for FFPE) was sheared in a Covaris S220 ultrasonicator (Covaris Inc., Woburn, MA, USA) and used for the construction of a library using CancerSCAN probes and an HSQ SureSelectXT reagent kit (Agilent Technologies Inc., Santa Clara, CA, USA) according to the manufacturers’ instructions. After the enriched exome libraries were multiplexed, the libraries were sequenced using the 100-bp paired-end mode of the TruSeq Rapid PE Cluster Kit and TruSeq Rapid SBS kit on the Illumina HiSeq 2500 sequencing platform (Illumina Inc., San Diego, CA, USA).
**Variant calling**

DNA sequence data were aligned to the reference human genome (hg19) using the MEM algorithm in BWA version 0.7.5 [9]. Duplicate read removal was performed using Picard version 193 and SAMtools version 0.1.18 [10]. Local alignment was optimized using the Genome Analysis Toolkit (GATK) version 3.1–1 [11]. We also used BaseRecalibrator from GATK for base recalibration based on known single nucleotide polymorphisms and indels from Mills, Single Nucleotide Polymorphism Database Build 138 (dbSNP138), 1000G gold standard, 1000G phase1, and Omni 2.5.

Variant calling was only performed in regions targeted in CancerSCAN™ version 2. We detected single nucleotide variants using two tools: MuTect version 1.1.4 and LoFreq version 0.6.1 [12,13]. Falsely detected variants were filtered out from abnormally aligned strand-biased and clustered reads using scripts developed in-house. ANNOVAR was used to annotate the detected variants with various resources, including dbSNP138, Catalogue of Somatic Mutations in Cancer (COSMIC), the Cancer Genome Atlas (TCGA), Exome Sequencing Project (ESP5400), 1000 Genomes Project, Exome Aggregation Consortium database (ExAC03), and the in-house Korean SNP database. Indels were detected with Pindel version 0.2.4 [14] and annotated using ANNOVAR. To filter out germline variants, we applied two algorithms: (1) except for hotspot mutations, variants with an allele frequency ≥97% were filtered out and (2) suspect germline variants were filtered out based on whether the allele frequency was ≥1% of the previously mentioned database or ≥3% of 480 samples from healthy Korean subjects. We used CancerSCAN™ software [15] to detect copy number variations (CNVs). In CancerSCAN™, the software “Depth of Coverage” in GATK version 3.1–1 was used to calculate sequencing coverage in each exon. The median of the mean coverage for the total number of exons was calculated and divided by the gained average for the normalization of the mean coverage of exons. The median value of normalized exons of pattern matched normal reference datasets (HapMap cell lines and FFPE tissues from normal patients) was used as a reference value. The normalized median value of exons for each patient was subsequently divided by the reference value and transformed to the binary logarithm. Tumor purity for adjusting CNVs was calculated using normalized coverage and B allele frequencies at all exons. Using normal samples, coverage of the sample was fitted, and subsequently, major ploidies and copy numbers in the neutral region were determined. Based on the inferred copy neutral region and major ploidies, tumor purity was calculated using the relationship between B allele frequencies and tumor ploidy data. Finally, we defined "copy number deletion" as a copy number of less than 0.7, and "copy number amplification" as a copy number of more than 4 using the above method.

**Tier classification and selection of potential targeted agents**

Identified molecular alterations were assessed using a multidisciplinary panel regarding biological relevance and potential druggability. Clinical or preclinical evidence was collected using ClinVar (https://www.ncbi.nlm.nih.gov/clinvar), My Cancer Genome (https://www.my cancergenome.org), Targeted Therapy Database (http://www.mmp.org), JAX-Clinical Knowledgebase (https://ckb.jax.org/), and the U.S. National Institute of Health clinical database (https://clinicaltrials.gov/). Variants and drug interactions were also manually reviewed in the literature. The molecular targets were tiered from 1 to 5: 1a, clinical evidence of the same alteration in the same disease; 1b, clinical evidence of the same alteration in a different disease; 2a, preclinical evidence of the same alteration in the same disease; 2b, preclinical evidence of the same alteration in a different disease; 3, consensus opinion; 4, actionable variants with drugs that are available in other countries for use in clinical trials or FDA-approved drugs.
for the same or other diseases but that are not available in Korea; and 5, no actionable genetic
variants.

Results
Patients and specimens
Fifty-five patients were enrolled between January 2016 and October 2018. The characteristics
of the patients and specimens are summarized in Table 1. The most frequent disease was neu-
roblastoma (n = 16, 29.1%). For 41 patients, tissue samples taken at recurrence were analyzed.
For the remaining patients (n = 14), primary tumors were sequenced because of tissue unavail-
ability at relapse. For 2 patients, sequencing could not be performed because of poor DNA
quality. The median time from tissue procurement to a variant calling-based clinical decision
was 29 d (range: 14 to 39), including 7 d (range: 2 to 15) for DNA preparation, 19 d (range: 7
to 29) for sequencing and bioinformatics analysis, and 2 d (range: 0 to 5) for deciding on the
targeted agents.

Results of targeted sequencing and tier classification
Detected genetic variants were illustrated in S2 Table. The average target depth for all samples
was 874.53× (SD = 234.03), and the median tumor mutation burden was 14.1 (range 0–43.7)
mutations/Mb. A total of 27 actionable alterations were detected in 20 patients (36.4%), and
the majority of actionable alterations were CNVs (Fig 1). In a patient with an inflammatory
myofibroblastic tumor, conventional fluorescence in situ hybridization (FISH) analysis

Table 1. Patients and specimens characteristics value.

| Age at enrollment, median (range, years) | 10.7 (0.8–20.7) |
| Sex, No. (%) |  |
| Male | 30 (54.5) |
| Female | 25 (45.5) |
| Diagnosis, No. (%) |  |
| Neuroblastoma | 16 (29.1) |
| Rhabdomyosarcoma | 9 (16.4) |
| Medulloblastoma | 5 (9.1) |
| High-grade gliomas | 4 (7.3) |
| Ewing sarcoma | 3 (5.5) |
| Hepatoblastoma | 3 (5.5) |
| Osteosarcoma | 3 (5.5) |
| Alveolar soft part sarcoma | 2 (3.6) |
| Wilms tumor | 2 (3.6) |
| Others* | 8 (14.5) |
| Tumor samples for analysis |  |
| Initial diagnosis | 14 (25.5) |
| Relapse/refractory/progression | 41 (74.5) |
| Type of sample |  |
| Paraffin-embedded tissue | 34 (61.8) |
| Fresh-frozen tissue | 21 (38.2) |

*Others include angiosarcoma, CNS embryonal tumor, desmoplastic small round cell tumor, EBV-associated
lymphoepithelioid carcinoma, epithelioid sarcoma, inflammatory myofibroblastic tumor, malignant rhabdoid tumor,
and yolk sac tumor.

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showed negative ALK rearrangement, but a TPM4-ALK translocation was detected by targeted sequencing.

Molecular alterations were tiered from 1 to 5; 3 variants were classified into tier 1, 8 variants were classified into tier 2, 2 variants were classified into tier 3, 10 variants were classified into tier 4, and the other 37 patients were classified into tier 5 with no actionable alteration. For the alterations in tiers 1–4, potential matched targeted agents were selected for each variant. The details of the genetic variants in tiers 1–4 are summarized in Table 2 along with the references used in the tier classification [16–30].

**Example of clinical application**

As an example of clinical application, a patient (No. 060) was diagnosed with stage 4, MYCN amplified neuroblastoma. He underwent induction chemotherapy, surgery, and tandem high-dose chemotherapy, but he relapsed immediately after the second high-dose chemotherapy. Our analysis of the relapsed tumor tissue sample revealed an ALK F1174L mutation and together with an ALK amplification (Fig 2A). Crizotinib, a 1st generation ALK inhibitor, has
been reported to have potency in in vitro studies with neuroblastoma cell lines [31], and it has been tested in a Phase I clinical trial for the treatment of pediatric solid tumors [32]. However, certain mutations such as **ALK**F1174L are known to have intrinsic crizotinib resistance, and Infarinato NR et al. demonstrated that lorlatinib (PF-06463922; Pfizer Oncology, Groton, CT, USA) had the potential to overcome crizotinib resistance against F1174L **ALK**-mutated xenograft tumors [29]. With this background, a clinical trial with lorlatinib in **ALK**-driven neuroblastoma is now being conducted by New Approaches to Neuroblastoma Therapy (NANT) Consortium (NCT03107988). Based on these facts, lorlatinib was used in a compassionate use program for this patient. After 8 weeks of treatment, the size of several metastatic lymph nodes along the left iliac chain decreased on computed tomography (CT) scan (largest size 1.6 cm → 1.0 cm). Positron emission tomography (PET) and metaiodobenzylguanidine (MIBG) scan also showed response (maximal SUV 6.6 → 2.4 and MIBG score 2 → 0). However, the tumor relapsed again after 16 weeks of treatment (Fig 2B).

**Discussion**

In this study, the possibility of the use of precision medicine for the management of refractory/relapsed pediatric solid tumors was assessed through a prospective study using a targeted sequencing panel. Following the advances in genomic technology and the development of targeted therapeutics, several studies have explored the feasibility of precision medicine in pediatric oncology [3–8]. In these studies, various genomic technologies were utilized, including targeted sequencing, array comparative genomic hybridization (aCGH) to detect copy number variation, whole exome sequencing, and RNA sequencing. Genetic alterations affecting clinical
decisions were reported in 31 to 73% of patients, depending on the study. The differences in the frequency of alterations are thought to be due to the various technologies used as well as the definition of actionable alterations used by each group. In our study, we used a targeted sequencing panel. Targeted panel sequencing has many advantages, including the relative simplicity of the method to detect known variants with high coverage and low complexity [33], thus making it easy to apply in clinical practice. With the targeted sequencing panel, tumor profiling was possible in 96.4% of patients, and the turnaround time of a median 29 d was acceptable for a clinical trial.

In our study, actionable alterations were found in 20 (36.4%) patients, and CNVs were the most frequent actionable targets. These results are consistent with the results of the Individualized Cancer Therapy (iCat) study by Harris MH, in which the most common actionable alteration leading to an iCat recommendation was a copy number alteration [4]. Somatic mutation frequency in pediatric cancer is much less than that of adult cancer; Lawrence MS et al showed that median frequency of non-synonymous mutations varied by more than 1,000-fold across cancer types, and pediatric cancers showed frequencies as low as 0.1/Mb [34]. Another study showed that tumor mutational burden increases significantly with age, showing a 2.4-fold
difference between age 10 and age 90 years [35]. In a recently published article that studied the landscape of genomic alterations across multiple childhood cancers, the mutation frequency and significantly mutated genes in pediatric cancer were markedly different compared to those reported previously for adult cancers [36]. These findings suggest that the genomic profiles of childhood cancer are different from those of adult tumors, so the approach to pediatric cancers needs to be different from that for adult cancers. The targeted sequencing panel used in this study was originally designed for adult cancers, so a panel designed specifically for pediatric cancer will be needed. The pediatric panel needs to include not only actionable genes but also genes used as diagnostic or prognostic markers for pediatric cancers. Moreover, the information obtained from targeted sequencing only provides a limited understanding of tumor biology, and further comprehensive research, including transcriptome, proteomic, and epigenomic analyses, will be needed to improve the detection of potential actionable alterations. In vitro drug screening with patient-derived cells represents another possible option to screen for potential drugs for use in precision medicine.

One of the clinical limitations of these approaches is that drug availability is limited even though actionable alterations were found. This is an inevitable problem in the pediatric population, especially in the Republic of Korea. Although considerable progress has been made, the development of drugs for childhood cancer is still in its early stages. Recently, the Innovative Therapies for Children with Cancer (ITCC) Consortium suggested a new adaptive design for early phase clinical trials in children and adolescents to facilitate drug development for childhood and adolescent cancers [37]. In parallel with these efforts, accessible clinical trials need to be expanded globally in order to increase the therapeutic options available to children and adolescents with relapsed or refractory disease.

We preferred samples taken at the time of relapse because there can be new additional mutations and clonal evolution at relapse. Some of patients in this study had samples taken at both diagnosis and relapse, and genomic changes in these patients were studied in our previous work [38]. In the previous study, 72.6% of SNVs in primary tumors were found in recurrent lesions, however, 27.2% of SNVs had newly occurred in recurrent tumors [38].

There are several limitations in our study. First of all, germline samples were not analyzed together with tumor samples, so interpretation of germline variants was limited in this study. Also, there are also limitations in interpreting variants of unknown significance detected, and more in-depth analysis including in silico analysis will be necessary in the future.

In conclusion, using a targeted sequencing panel to discover actionable alterations in relapsed refractory pediatric tumors was practical and feasible. More intensive genomic studies and efforts to increase access to potential drugs are needed to improve the application of precision medicine in pediatric cancer.

Supporting information

**S1 Table. List of target genes.** Target genes included in targeted sequencing panel (CancerS-CAN™) were listed.
(XLSX)

**S2 Table. Genetic variants identified were listed.**
(XLSX)

**S1 Data. Data set.**
(XLSX)
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