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ONCOGENE MUTATION PROFILING OF PEDIATRIC SOLID TUMORS REVEALS SIGNIFICANT SUBSETS OF EMBRYONAL RHABDOMYOSARCOMA AND NEUROBLASTOMA WITH MUTATED GENES IN GROWTH SIGNALING PATHWAYS

Neerav Shukla[1], **Nabahet Ameur**[2], **Ismail Yilmaz**[3], **Khedoudja Nafa**[3], **Chyau-Yueh Lau**[3], **Angela Marchetti**[3], **Laetitia Borsu**[3], **Frederic G. Barr**[4], and **Marc Ladanyi**[2,3]

[1]Department of Pediatrics, Memorial Sloan-Kettering Cancer Center, New York, NY, 10065

[2]Human Oncology and Pathogenesis Program, Memorial Sloan-Kettering Cancer Center, New York, NY, 10065

[3]Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York, NY, 10065

[4]Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA 19104

Abstract

Compared to the numerous broad screens for oncogene mutations in adult cancers, very few have been performed in pediatric solid tumors. To identify novel mutations and potential therapeutic targets in pediatric cancers, we performed a high-throughput Sequenom-based analysis in large sets of several major pediatric solid cancers, including neuroblastoma (NB), Ewing sarcoma (ES), rhabdomyosarcoma (RMS), and desmoplastic small round cell tumor (DSRCT).

Experimental Design—We designed a highly multiplexed Sequenom-based assay to interrogate 275 recurrent mutations across 29 genes. Genomic DNA was extracted from 192 NB, 75 ES, 89 RMS, and 24 DSRCT samples. All mutations were verified by Sanger sequencing.

Results—Mutations were identified in 13% of NB samples, 4% of ES samples, 21.1% of RMS samples, and no DSRCT samples. *ALK* mutations were present in 10.4% of NB samples. The remainder of NB mutations involved the *BRAF*, *RAS*, and *MAP2K1* genes and were absent in samples harboring *ALK* mutations. Mutations were more common in embryonal RMS (ERMS) samples (28.3%) than alveolar RMS (ARMS) (3.5%). In addition to previously identified *RAS* and *FGFR4* mutations, we report for the first time *PIK3CA* and *CTNNB1* (Beta-Catenin) mutations in 4.9% and 3.3% of ERMS, respectively.

Conclusions—In ERMS, ES, and NB, we identified novel occurrences of several oncogene mutations recognized as drivers in other cancers. Overall, NB and ERMS contain significant subsets of cases with non-overlapping mutated genes in growth signaling pathways. Tumor profiling can identify a subset of pediatric solid tumor patients as candidates for kinase inhibitors or RAS-targeted therapies.

Keywords

mutation; rhabdomyosarcoma; neuroblastoma; Ewing sarcoma; desmoplastic small round cell tumor

Correspondence and reprint requests: Marc Ladanyi, MD, Department of Pathology, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, NEW YORK, NY 10065, Tel: 212 639 6369, Fax: 212 717 3515, ladanyim@mskcc.org.

Introduction

Many different tumor types harbor somatic gene mutations which contribute to tumor development and can also serve as promising therapeutic targets. Examples of therapeutically relevant mutations include *KIT* and *PDGFRA* mutations in gastrointestinal stromal tumors, *EGFR* mutations in lung adenocarcinomas, and *BRAF* mutations in melanoma.(1-4) More recently, activating *ALK* mutations have been identified in a subset of neuroblastomas, demonstrating the presence of therapeutically susceptible mutations in pediatric tumors.(5, 6)

Numerous large scale gene profiling studies have been performed to identify novel mutations in various tumor types.(7-9) However, broad mutational profiling studies have not been performed on a large cohort of NB and pediatric sarcoma samples. Furthermore, although *ALK* harbors recurrent mutations in neuroblastoma, *ALK* mutated tumors account for less than 15% of neuroblastoma cases (5), raising the possibility of alternative signaling gene mutations in additional subsets of this cancer.

The Sequenom MassARRAY technology utilizes a mass spectrometry-based genotyping approach which allows for more sensitive mutational analysis compared to traditional Sanger sequencing.(8) This platform also performs well using DNA extracted from archived paraffin embedded material. Furthermore, highly multiplexed PCR assays allow for efficient high-throughput screening of large tumor sample sets. (9)

In order to identify novel mutations in pediatric tumors, we utilized the Sequenom MassARRAY platform to perform a high-throughput sequencing analysis interrogating 275 point mutations across 29 oncogenes in large sample sets of four tumor types: NB, ES, RMS, and DSRCT. In addition to known recurrent somatically mutated genes, we included an extended panel of *BRAF* and *PTPN11* mutation assays, based on the notion that known germline point mutations in these genes, defined in a constellation of conditions grouped together as *RAS/MAPK* syndromes (10, 11), might also occur as sporadic somatic mutations. Patients with these genetic disorders have an increased risk of pediatric neoplasia including rhabdomyosarcoma and neuroblastoma.(12, 13) The results of our mutation screen show that tumor mutation profiling can identify a subset of patients with NB and pediatric sarcomas who may be candidates for novel *RAS*, *MEK*, and *PIK3CA* inhibitors.

Methods

Tumor Samples

All frozen tumor samples and formalin-fixed paraffin embedded (FFPE) samples were procured at Memorial Sloan-Kettering Cancer Center (MSKCC) under IRB-approved protocols. Genomic DNA was extracted using the Qiagen DNeasy kit according to the manufacturer's directions. DNA from 192 NB samples, 75 ES, 89 RMS, and 24 DSRCT samples and cell lines was collected. Cell lines used in the study are listed in Supplemental Table S1. Testing for *EWSR1* and *PAX/FKHR* rearrangements was performed by RT-PCR or FISH, as previously described (14).

Curation of Oncogene Point Mutations

We queried the Sanger Institute COSMIC online database for recurrent non-synonymous point mutations known to occur in different human cancer types. Mutations were selected based on their susceptibility as a therapeutic target and mutation frequency. Furthermore, an extended list of *BRAF* and *PTPN11* mutations was generated based on known germline mutations in *RAS/MAPK* syndromes (10, 11). Insertion and deletion type mutations were not

included. A total of 275 point mutations (Supplemental Table S2) across 29 genes were selected for interrogation (Table 1). Input sequence files for assay design were generated by the MSKCC Bioinformatics Core Facility.

Sequenom Based DNA Sequencing

For these assays we used the MassARRAY system (Sequenom), which is based on matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Genotyping by this method relies on the principle that mutant and wild-type alleles for a given point mutation produce single-allele base extension reaction products of a mass that is specific to the sequence of the product. Mutation calls are based on the mass differences between the wild-type product and the mutant products as resolved by MALDI-TOF MS. Amplification and extension primers were designed using Sequenom Assay Designer v3.1 software to target the curated list of mutations. Amplification primers were designed with a 10mer tag sequence to increase their mass so that they fall outside the range of detection of the MALDI-TOF MS. The sequences of primers are shown in Supplemental Table S3.

The initial PCR amplification was performed in a 5-μL reaction mixture containing 10 to 20 ng of DNA/1.25x buffer; 1.625 mmol/L MgCl2; 500 μmol/L deoxynucleotide triphosphate (dNTP); 100 nmol/L from each primer; and 0.5 U HotStar TaqDNA Polymerase (Qiagen) under the following conditions: 95°C (15 minutes); 95°C (20 seconds): 56°C (30 seconds); and 72°C (60 seconds) for 45 cycles, and a final extension phase at 72°C (3 minutes). Remaining unincorporated dNTPs were dephosphorylated by adding 2 μL of a shrimp alkaline phosphatase cocktail containing 1.53 μL of water, 0.17 μL of reaction buffer (Sequenom), and 0.3 μL of shrimp alkaline phosphatase (Sequenom). The unincorporated dNTPs were then placed in a thermal cycler under the following conditions: 37°C for 40 minutes, 85°C for 5 minutes, and then held at 4°C indefinitely. A single-base extension reaction was then performed in a 2-μL TypePLEX reaction mix (Sequenom) consisting of 0.72 μL water; 0.20 μL TypePLEX 10x buffer (Sequenom); 0.10 μL TypePLEX terminator mix (Sequenom); 0.94 μL extension primer mixture; and 0.04 μL TypePLEX enzyme (Sequenom). Thermal cycling was performed under the following conditions: 94°C for 30 seconds followed by 40 cycles of (94°C for 5 seconds, 5 cycles of 52°C for 5 seconds and 80°C for 5 seconds), then at 72°C for 3 minutes, and was finally held at 4°C indefinitely. The reaction mixture was then desalted by adding 16 μL of water and 6 mg of cationic resin mixture, SpectroCLEAN (Sequenom), and placed in a rotating shaker for 30 minutes. Completed genotyping reactions were spotted in nanoliter volumes onto a matrix-arrayed silicon SpectroCHIP with 384 elements using the MassARRAY Nanodispenser (Sequenom). SpectroCHIPs were analyzed using the Autoflex MALDI-TOF MS (Bruker AXS, Madison, WI), and the spectra were processed using SpectroACQUIRE software (Sequenom) in real time. Genotype calls are automatically generated using complex mathematical algorithms according to the peak heights, noise-to-peak-height ratio, area under the curve, and anchoring of the peaks. Results are then linked to plate information created in MassARRAY Typer 4.0 software (Sequenom). Individual calls are manually reviewed and finalized.

Whole Genome Amplification (WGA)

WGA was performed on 62 RMS samples and 74 EWS samples for sequencing of *FGFR4* and *CTNNB1*. For frozen tissue, the Repli-G Midi (Qiagen, Valencia, CA) was used to amplify 100 ng of genomic DNA. WGA DNA was assessed by Picogreen quantification followed by PCR amplification with two control amplicons. For FFPE samples, The Sigma GenomePlex (Sigma Aldrich) was used to amplify 100 ng of genomic DNA. WGA DNA was assessed by Picogreen quantification followed by PCR amplification with two control amplicons.

PCR Amplification and DNA Sequencing of WGA Samples

All exons of *FGFR4* and exon 3 of *CTNNB1* were amplified as amplicons of 500 bp or less, covering the exonic regions plus at least 50 bp of intronic sequences (Supplemental Table S4). M13 tails were added to the primers to facilitate Sanger sequencing. RMS samples were evaluated for *FGFR4* and *CTNNB1* mutations. ES samples were evaluated for CTNNB1 mutations. PCR reactions were carried out in 384 well plates, in a Duncan DT-24 water bath thermal cycler, with 10 ng of whole genome amplified DNA as template, using a touchdown PCR protocol with PCR reactions were carried out in 384 well plates, in a Duncan DT-24 water bath thermal cycler, with 10 ng of whole genome amplified DNA (Repli-G Midi, Qiagen) as template, using a touchdown PCR protocol with HotStart Kapa Fast Taq (Kapa Biosystems). The touchdown PCR method consisted of : 1 cycle of 95°C for 5 min; 3 cycles of 95°C for 30 sec, 64°C for 15 sec, 72°C for 30 sec; 3 cycles of 95°C for 30 sec, 62°C for 15 sec, 72°C for 30 sec; 3 cycles of 95°C for 30 sec, 60°C for 15 sec, 72°C for 30 sec; 37 cycles of 95°C for 30 sec, 58°C for 15 sec, 72°C for 30 sec; 1 cycle of 70°C for 5 min. Templates were purified using AMPure (Agencourt Biosciences, Beverly, MA). The purified PCR reactions were split into two, and sequenced bidirectionally with M13 forward and reverse primer and Big Dye Terminator Kit v.3.1 (Applied Biosystems, Foster City, CA), at Agencourt Biosciences. Dye terminators were removed using the CleanSEQ kit (Agencourt Biosciences), and sequence reactions were run on ABI PRISM 3730xl sequencing apparatus (Applied Biosystems, Foster City, CA). Mutations were detected using an automated detection pipeline at the MSKCC Bioinformatics Core. Bi-directional reads and mapping tables (to link read names to sample identifiers, gene names, read direction, and amplicon) were subjected to a QC filter which excludes reads that have an average phred score of < 10 for bases 100-200. Passing reads were assembled against the reference sequences for each gene, containing all coding and UTR exons including 5Kb upstream and downstream of the gene, using command line Consed 16.0 (PMID: 9521923). All traces for mutation calls were manually reviewed and validated with original non-WGA DNA.

PCR Amplification and DNA Sequencing for Mutation Validation

All identified mutations were verified by standard Sanger sequencing. Forward and reverse primers are listed in Supplemental Table S5. Each PCR reaction was performed in a 50-μL volume mixture containing 100 ng of genomic DNA; forward and reverse primers (20 pmol each); 200 μM of each dNTP; 1.5 mmol/L $MgCl_2$; 1X Qiagen PCR buffer containing 1.5 mmol/L $MgCl_2$ and 2.5 units of HotStarTaq DNA polymerase (Qiagen). The PCR amplification was carried out under the following conditions: 1 cycle at 95°C (15 minutes); 40 cycles at 94°C (30 seconds), at 60°C (30 seconds), and at 72°C (60 seconds); and a final extension step at 72°C (10 minutes). Amplified products were purified using Spin Columns (Qiagen) and sequenced in both directions using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), according to the manufacturer's protocol, on an ABI3730xl running ABI Prism DNA Sequence Analysis Software (Applied Biosystems).

Results

Mutations were identified by Sequenom analysis in 13% (25/192) of NB samples, 4% (3/75) of ES samples, 20.2% of RMS samples (18/89), and 0% of DSRCT samples (Table 2). Clinical information on the patients with mutated samples is provided in Supplemental Table S6. Mutations were found in 28.3% (17/60) of embryonal RMS (ERMS) tumors, with 13/60 (21.7%) identified by Sequenom analysis and the remainder identified by direct sequencing of *FGFR4* (described below). Mutations were observed in 3.5% (1/29) of alveolar RMS (ARMS) samples. The majority of NB mutations were activating *ALK* mutations (Table 3), accounting for 10.4% of the tumor samples, a proportion consistent

with previous reports (15). The remainder of NB mutations involved *BRAF*, *RAS*, and *MAP2K1* genes and were not seen in samples harboring *ALK* mutations.

RAS family mutations were found in a subset of NB, RMS, and ES samples. 11.7% (7/60) of ERMS samples, 3.5% (1/29) of ARMS samples, 1% (2/192) of NB samples, and 1.3% (1/75) of ES samples harbored RAS mutations on our panel.

BRAF mutations were identified in 1% (2/192) of NB samples, 1.7% (1/60) of ERMS samples, and 1.3% (1/75) of ES samples. The ERMS and ES samples harbored the activating $BRAF^{V600E}$ mutation. One NB sample carried the $BRAF^{V600E}$ mutation, while the other carried the less common $BRAF^{D594V}$ mutation. A $MAP2K1^{K57N}$ mutation was identified in one NB sample.

PIK3CA mutations were identified in 5% (3/60) of ERMS cases, but were not seen in any other tumor type in our screen (Figure 1). Two of the 3 mutations were in the helical domain of the gene (E542K and E545K). The other mutation was located in the kinase domain (H1047R).

CTNNB1 mutations were identified in 1.3% (1/75) of ES samples, and 3.3% (2/60) of ERMS samples (Figure 2). One of the ERMS samples contained both an *NRAS* and a *CTNNB1* gene mutation.

A *PTPN11* mutation was identified in 1.7% (1/60) ERMS samples. The tumor was from a patient diagnosed with Neurofibromatosis type 1. Sequencing of normal tissue from this patient confirmed the *PTPN11* mutation to be somatic.

Full sequencing of the coding exons of *FGFR4* and beta-catenin was performed in 43 ERMS and 19 ARMS samples. Similarly, full sequencing of beta-catenin was performed on 76 ES tumor samples. The V550L *FGFR4* mutation was identified in 9.3% (4/43) ERMS tumors and none of the ARMS tumors. Sequencing of matched normal tissue, available for 2 of the 4 samples, confirmed the *FGFR4* mutations to be somatic in both cases. Of the remaining 2 *FGFR4*-mutated samples, one lacked corresponding normal tissue and the other was a cell line (RMS 559). Additional *CTNNB1* mutations, aside from those detected by the Sequenom assay, were not found in any of the RMS or ES samples.

Discussion

Multiple large scale cancer genomics efforts are underway to better characterize tumors and to help identify new therapeutic targets but few of these include pediatric solid tumors. Several genes have been examined for mutations in various pediatric tumor types (5, 16-27) but the present study represents the most expansive “hotspot” mutation profiling for these tumor types that has been reported to date. Here, we used a mass spectrometry-based platform to survey recurrent point mutations in 29 cancer genes in 380 cases of 4 major pediatric solid cancers.

The frequency of mutations found in our study was lower than in most adult onset carcinomas. Given the embryonal origin of these pediatric cancers, it is also possible that different genes are recurrently mutated in these tumor types. More comprehensive approaches such as whole exome sequencing would be needed to identify such mutations. However, a significant proportion of ERMS tumor samples were found to harbor various targetable mutations from our focused screen (Figure 3). *RAS* mutations are known to occur in a minority of ERMS patients.(17, 28) More recently, Paulson et al identified *RAS* mutations in 11/26 (42%) of ERMS samples. Furthermore, they identified *NF1* gene deletions in 15% of ERMS samples, representing an alternative mechanism of *RAS* pathway

activation.(29) In our study, 7 cases of ERMS harbored a *RAS* mutation. A codon 61 mutation of *NRAS* was found in 4 samples, including the RD cell line, as previously reported.(30) Two cases had *KRAS* codon 12 mutations. The ERMS cell line SMS-CTR was found to have an *HRAS* Q61K mutation. One of the 29 ARMS samples was found to have a *RAS* mutation. It was the sole mutation found in our cohort of ARMS samples. Our mutation data suggests that overall, *RAS* mutations are more typical of translocation-negative RMS but this difference may require a larger sample set to confirm statistically.

To our knowledge, *RAS* mutations have not been described in NB or ES. Whereas two studies have reported completely negative results(16, 19), another study described a rare codon 59 *NRAS* mutation in a NB cell line, which was not seen when the original tumor DNA was tested.(18) Our study shows that *RAS* mutations may play a role in rare cases of ES and NB.

BRAF point mutations have been studied in various pediatric solid tumors. For instance, mutations have been described in a significant proportion of low-grade pediatric astrocytomas.(31) A $BRAF^{V600E}$ mutation has been previously identified in the ES cell line A673 as well as in 2 RMS cell lines not used in our study.(1) However *BRAF* mutations have not been reported in NB cases.(21) Our study findings indicate that *BRAF* mutations are rare in these tumor types, but may play an oncogenic role in a small subset of sarcoma and NB patients. The $BRAF^{V600E}$ mutation was identified in a single ERMS tumor sample. Furthermore, 2/190 NB tumor samples harbored *BRAF* mutations, and another NB was found to have an activating mutation of *MAP2K1* (*MEK1*), indicating a role of *MAPK/ERK* pathway activation in a subset of NB patients.

Unlike adult-onset tumors, mutations in the *PIK3CA* gene have not been commonly reported in pediatric tumor studies. One report of *PIK3CA* mutations in NB samples found 2 point mutations in 69 samples. However, neither of these substitutions were the frequent gain-of-function mutations (E542K, E545K, H1047R) often seen in other solid tumor types. Therefore, the effect on kinase activity of these mutations is unknown.(24) We did not identify any *PIK3CA* mutations in our larger set of NB cases.

Recently, 18% of myxoid/round-cell liposarcomas were found to have *PIK3CA* mutations localized to the helical and kinase domains, and these conferred a worse prognosis than wild-type *PIK3CA* within the same patient population.(32) *PIK3CA* mutations have not been previously reported in pediatric sarcomas. We identified *PIK3CA* mutations in 3/60 (5%) of ERMS samples, including two mutations in the helical domain and one in the kinase domain. Both mutations are known to be oncogenic.(33) Activation of *AKT* through the insulin-like growth factor pathway has been well established in RMS.(34-36) Our data suggest the possibility of an additional role of activating *PIK3CA* mutations in activating AKT in some ERMS patients. Furthermore, all 3 *PIK3CA*-mutant ERMS patients died of relapsed disease suggesting a more aggressive phenotype than is typical for ERMS. An expanded RMS sample set will be necessary to rigorously assess the impact of *PIK3CA* mutations on outcomes. In vitro evaluation of novel *PIK3CA* inhibitors in RMS cell lines along with efforts to establish RMS cell lines harboring *PIK3CA* mutations are currently in progress.

CTNNB1 (Beta-catenin) mutations have been identified in several embryonal pediatric tumor types. Activating *CTNNB1* mutations occur in 10-15% of medulloblastoma cases and seem to define a distinct subgroup with improved overall outcomes.(27) *CTNNB1* mutations have also been well characterized in hepatoblastomas and pediatric Wilms tumors.(20, 37) In a recent report, an evaluation of the *CTNNB1* gene in 14 RMS samples did not identify any gene mutations.(38) In our screen, we identified a single mutated ES sample, and 2

RMS cases with mutations. Due to the heterogeneity of mutations in exon 3 of this gene, many of which were not included in our Sequenom panel, Sanger sequencing of exon 3 was performed in ES and RMS samples but this did not identify any additional mutations. However, these findings identify a small subset of RMS patients in which activation of the *CTNNB1* pathway may play a role in tumor development. Intriguingly, both RMS patients with *CTNNB1* mutations are alive and disease free for at least 6 years since diagnosis, including one patient diagnosed with stage 4 disease. This raises the possibility of improved outcomes in RMS patients with *CTNNB1* mutations, similar to the patients with *CTNNB1*-mutated medulloblastoma.

There is an increased incidence of RMS and other childhood cancers in genetic syndromes with germline *RAS/MAPK* pathway mutations, including Cardio-facio-cutaneous Syndrome, Costello Syndrome, and Noonan Syndrome. Along with *RAS* genes, *PTPN11* and *BRAF* are two of the most frequently mutated genes in this group of syndromes, with the germline mutations overlapping only partly with recurrent somatic mutations in these genes.(10, 11, 13) Therefore, we interrogated an extensive range of possible mutations in these genes to assess the possibility that mutations previously reported as germline may occasionally be acquired in sporadic RMS cases. However, we identified only one *PTPN11* and one *BRAF* mutation in our survey of RMS samples.

FGFR4 mutations were recently identified in 7.5% of primary RMS tumors with mutations involving amino acids 535 and 550 leading to increased receptor phosphorylation and tumor progression. Mutations involving these amino acids were also most susceptible to *FGFR4* inhibition.(39) We identified *FGFR4* mutations in 4 out of 63 tumors tested (6.3%). All 4 samples were ERMS, including the cell line RMS559. Interestingly, all 4 mutations led to a V550L amino acid change.

Our panel included several recently described oncogenes which have not been extensively evaluated in pediatric solid tumors, including the *IDH* and *GNAQ* genes. *IDH1* and *IDH2* mutations were initially identified as frequently mutated in adult onset gliomas(40), and soon after were identified in a significant percentage of cytogenetically normal AML samples.(41) Frequent mutations in the *GNAQ* gene have been recently identified in melanocytic tumors leading to activation of the *MAPK* pathway.(42) A large analysis of glial, epithelial, and stromal type adult onset tumors did not identify any mutations in non-melanocytic tumors.(43) Our assay performed a mutational survey of the primary hotspots of these genes; namely R132 and R172 of *IDH1* and *IDH2*, and Q209 of *GNAQ*. We did not identify any mutations in either gene in our samples. Interestingly, *IDH* mutation studies of pediatric gliomas reveal that they occur in children 14 years of age or older, and not in younger children.(44) Similarly, *IDH* mutations are not characteristic of pediatric AML(45), indicating that these mutations may be much more likely to exist in adult-onset tumor types. Recently, a large mutational survey of the *IDH* gene in chondrosarcomas and chondromas revealed mutations in over 50% of cases, with the most frequent mutation involving the R132 codon.(46) Further mutational studies in other sarcoma types are warranted.

This analysis identified several novel occurrences of known oncogene mutations in pediatric tumors including *PIK3CA* and *CTNNB1* mutations in RMS, *CTNNB1* mutations in ES, and *RAS, BRAF, and MAP2K1* mutations in NB. No mutations were identified in a relatively large sample size of DSRCT. The paucity of mutations identified in ES samples, and the absence of mutations identified in DSRCT samples may be a function of the characteristic aberrant transcription factors generated by the specific translocations found in the two tumor types. The broad transcriptional dysregulation caused by these chimeric proteins may affect multiple oncogenic pathways, reducing the selective pressure for additional oncogenic mutations for tumor formation and progression. However, whole genome mutational studies

on ES and DSRCT need to be performed to confirm this hypothesis. A significant proportion of ERMS harbor oncogenic mutations. Including *FGFR4* mutations, 28.3% (17/60) of ERMS samples had an identifiable oncogenic mutation (figure 3).

Several mutations identified are of present interest in terms of targeted agents in clinical development. *MEK* inhibition has been recognized as an effective modality against melanomas harboring *BRAF* mutations.(47) Various *MEK* inhibitors are currently in phase III trials for patients with *BRAF*-mutant melanoma. Similarly, many different *PIK3CA* inhibitors are under development with numerous early phase studies open for adult-onset tumor types with corresponding mutations.(48) *ALK* inhibition has been recognized as effective across various tumor cell lines with *ALK* mutations or rearrangements.(49) The *ALK* inhibitor crizotinib is currently in phase I/II studies for refractory pediatric solid tumor patients, and phase III studies for lung cancer patients harboring *ALK* rearrangements. Recent studies have also demonstrated the activity of *MEK* inhibitors against a subset of *RAS*-mutant tumors.(50) As more agents inhibiting mutated oncoproteins and their associated downstream signaling pathways become available, mutational genotyping of pediatric solid tumors such as RMS will serve as an important tool for identifying targeted therapy options for individual patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of translational relevance

To identify novel mutations and potential therapeutic targets in pediatric cancers, we performed a high-throughput screen to interrogate 275 recurrent mutation sites across 29 cancer genes in 192 neuroblastomas (NB), 75 Ewing sarcomas (ES), 89 rhabdomyosarcomas (RMS), and 24 desmoplastic small round cell tumors (DSRCT). In embryonal RMS (ERMS), ES, and NB, we identified novel occurrences of several oncogene mutations recognized as drivers in other cancers, including for the first time *PIK3CA* and *CTNNB1* (beta-catenin) mutations in ERMS. Overall, NB and ERMS contain significant subsets of cases with non-overlapping mutated genes in growth signaling pathways. In particular, a substantial minority of ERMS show mutations in the RAS pathway. As more agents inhibiting mutated oncoproteins and their associated downstream signaling pathways become available, mutational genotyping of pediatric solid tumors such as RMS will serve as an important tool for identifying targeted therapy options for individual patients.

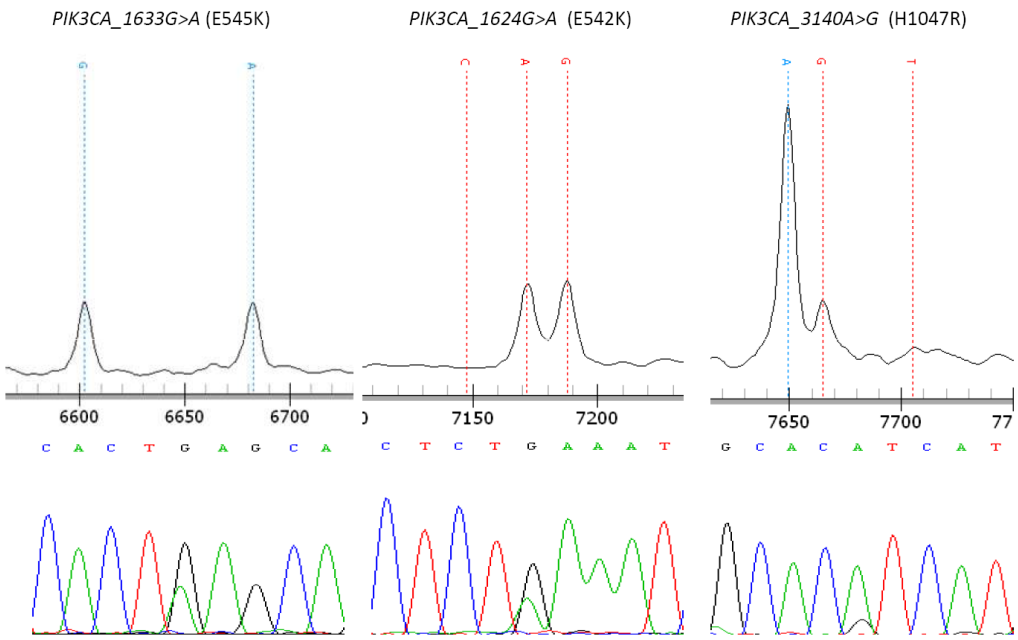

Figure 1. *PIK3CA* mutations in rhabdomyosarcoma

3 mutations were identified by Sequenom analysis and verified by direct sequencing. E542K and E545K mutations involve the helical domain of PIK3CA. The H1047R mutation involves the kinase domain of PIK3CA.

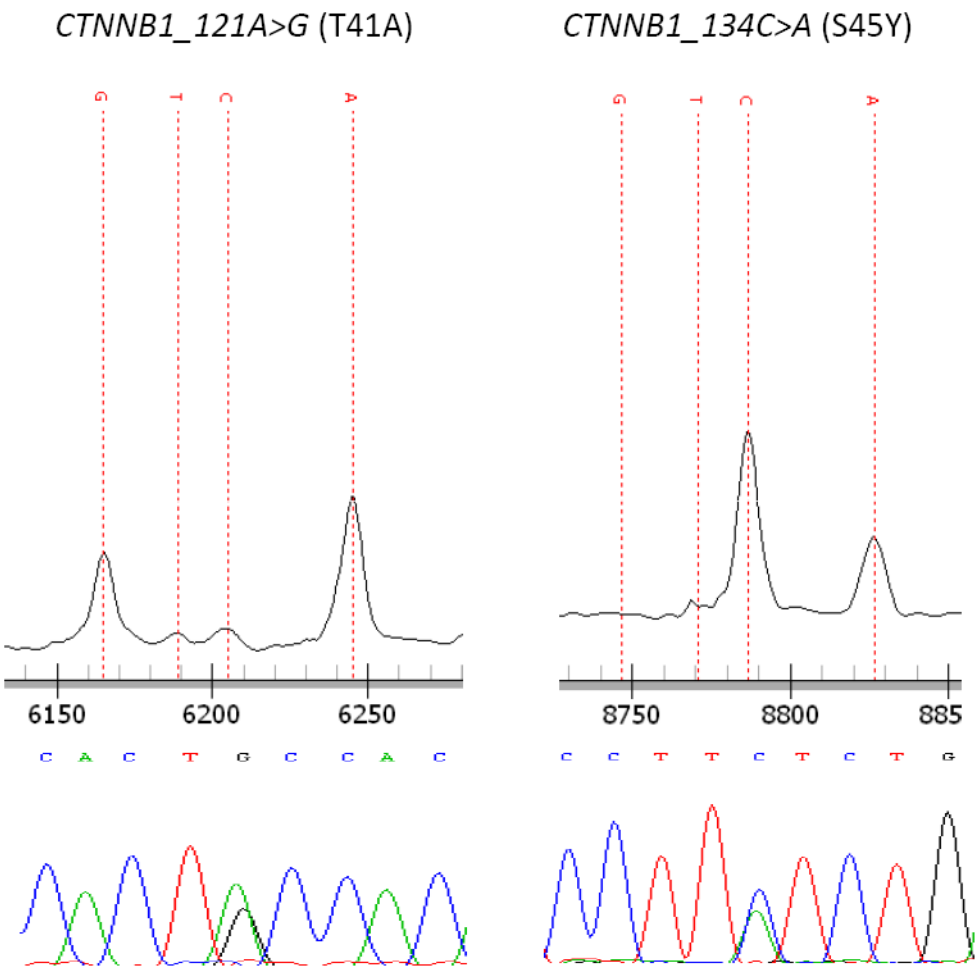

Figure 2. ***CTNNB1*** **mutations in rhabdomyosarcoma**

2 mutations were identified by Sequenom analysis and verified by direct sequencing.

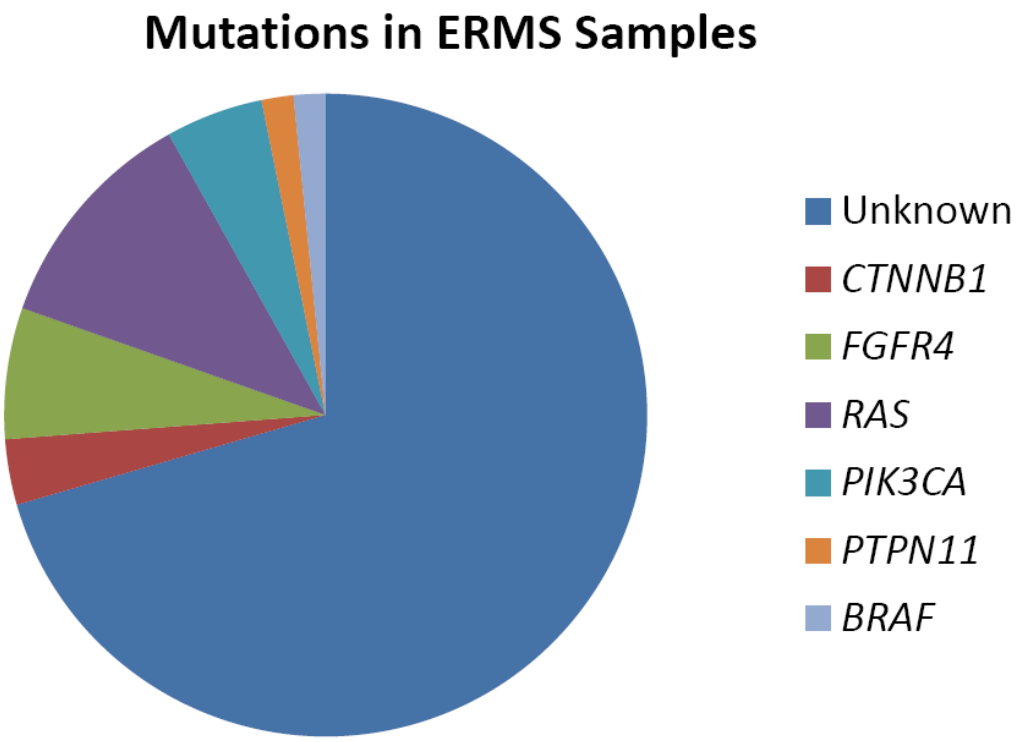

Figure 3. Oncogenic mutations identified in ERMS samples

Mutations in one of 6 known cancer genes were identified in approximately 28% of all ERMS samples tested.

Table 1

Oncogenes included in the Sequenom panel

The panel included 29 genes with known recurrent oncogenic mutations. 275 nucleotide changes were interrogated.

AKT1	FGFR3	MET
AKT2	FLT3	NOTCH1
AKT3	GNAQ	NRAS
ALK	HRAS	PDGFRA
BRAF	IDH1	PIK3CA
CDK4	IDH2	PIK3R1
CTNNB1	JAK2	PTPN11
EGFR	KIT	RET
ERBB2	KRAS	SMO
FGFR2	MAP2K1	

Table 2

Summary of identified mutations

Mutations were identified in three of the four tumor types: A) Rhabdomyosarcoma. FGFR4 mutations were identified by direct sequencing. B) Neuroblastoma (ALK mutations are not included) C) Ewing Sarcoma. No mutations were identified in any Desmoplastic Small Round Cell tumors.

A. Rhabdomyosarcoma (n=89)		
Sample ID	**Histology**	**Gene Mutation**
R60	ERMS	BRAF_1799T>A (V600E)
R11	ERMS	CTNNB1_121A>G (T41A), NRAS_181C>A (Q61K)
R7	ERMS	CTNNB1_134C>A (S45Y)
R13	ERMS	FGFR4_1648G>C (V550L)
R31 (RMS559 cell line)	ERMS	FGFR4_1648G>C (V550L)
R36	ERMS	FGFR4_1648G>C (V550L)
R6	ERMS	FGFR4_1648G>C (V550L)
R51 (SMS-CTR cell line)	ERMS	HRAS_181C>A (Q61K)
R61	ERMS	KRAS_34G>T (G12C)
R50	ERMS	KRAS_35G>A (G12D)
R71	ARMS	KRAS_38G>A (G13D)
R44	ERMS	NRAS 181C>A (Q61K)
R29	ERMS	NRAS_181C>A (Q61K)
R35 (RD cell line)	ERMS	NRAS_183A>T (Q61H)
R1	ERMS	PIK3CA_1624G>A (E542K)
R43	ERMS	PIK3CA_1633G>A (E545K)
R48	ERMS	PIK3CA_3140A>G (H1047R)
R33	ERMS	PTPN11_226G>C (E76Q)

B. Neuroblastomas (n=192)			
Sample ID	***MYCN* Amplification**	***ALK* Mutation**	**Gene Mutation**
NB32	no	no	BRAF_1783T>C (F595L)
NB158	no	no	BRAF_1799T>A (V600E)
NB23	yes	no	KRAS_35G>T (G12V)
NB139	no	no	MAP2K1_171G>T (K57N)
NB151	no	no	NRAS_181C>A (Q61K)

C. Ewing sarcomas (n=75)		
Sample ID	**Translocation**	**Gene Mutation**
ES2 (A673 cell line)	EWS-FLI1	BRAF_1799T>A (V600E)
ES9	EWS-FLI1	CTNNB1 133_135delTCT (S45del)
ES17	EWS-ERG	NRAS_181C>A (Q61K)

Table 3
ALK mutations detected by the Sequenom panel

ALK mutations were identified in 20/192 (10.4%) NB samples. No additional mutations were identified in this cohort of tumors. ALK mutations were not identified in RMS, ES, or DSRCT samples.

Neuroblastoma Sample	ALK Mutation
NB58	F1174C
NB10	F1174L
NB174	F1174L
NB191	F1174L
NB3	F1174L
NB30	F1174L
NB39	F1174L
NB54	F1174L
NB81	F1174L
NB83	F1174L
NB188	F1174L
NB62	F1245C
NB12	F1245V
NB109	R1275Q
NB129	R1275Q
NB134	R1275Q
NB135	R1275Q
NB171	R1275Q
NB2	R1275Q
NB44	R1275Q