Next-Generation Sequencing Identifies Potential Actionable Targets In Pediatric Sarcomas

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Research Article

Keywords: Pediatric sarcomas, Next-generation sequencing, Precision medicine, Clinical translation, Targeted drugs

Posted Date: December 23rd, 2020

DOI: https://doi.org/10.21203/rs.3.rs-124503/v1

Abstract

BACKGROUND Pediatric bone and soft-tissue sarcomas represent 13% of all pediatric malignancies. International contributions to introduce next-generation sequencing (NGS) approaches into clinical application are currently developing. We present the results from the Precision Medicine program for children with sarcomas at a reference center.

RESULTS Samples of 70 pediatric sarcomas were processed for histopathological analysis, RT-PCR and NGS with a consensus gene panel. Pathogenic alterations were reported and if existing, targeted recommendations were translated to the clinic. Seventy pediatric patients with sarcomas from 10 centers were studied. Median age was 11.5 years (range 1-18). Twenty-two (31%) had at least one pathogenic alteration by NGS. Thirty pathogenic mutations in 18 different genes were detected amongst the 22 patients. The most frequent alterations were found in *TP53*, followed by *FGFR4 and CTNNB1.* Eighteen actionable variants were detected and six patients received targeted treatment observing a disease control rate of 78%. Extrapolating the results to the whole cohort, 23% of the patients would obtain clinical benefit from this approach.

CONCLUSIONS Pediatric sarcomas have a different genomic landscape when compared to adult cohorts. Incorporating NGS targets into pediatric sarcomas' therapy is feasible and allows personalized treatments with clinical benefit in the relapse setting.

Introduction

Pediatric sarcomas account for over 20% of all pediatric solid malignant cancers and represent 13% of all pediatric malignancies[1]. They also contribute substantially to cancer-related mortality and morbidity. With more than over 70 histologic subtypes, sarcomas can arise from a primitive mesenchymal cell from almost every tissue in the human body and are classified into two main groups: soft tissue sarcomas (STS) and bone sarcomas (BS). The highest incidence rates in children are reported amongst rhabdomyosarcoma (RMS), osteosarcoma and Ewing's sarcoma (EWS). Although each subtype has a different phenotype and genetic profile, they are classified into two molecular groups: a *genetically complex* group with a high mutational burden and complex karyotype, and a *genetically simple* group containing a single and disease-specific translocation, amplification or mutation with a silent genomic background[2]. Most pediatric sarcomas are included in the second group as they are mostly characterized by chromosomal translocations that result in hybrid genes acting as drivers that are critical for sarcomagenesis[3].

Pediatric RMS protocols, currently classify this tumor based on the presence of *PAX/FOXO1* translocation and distinguish between fusion positive or fusion negative RMS[4]. The genetic profile of EWS is dominated by the driving reciprocal chimeric translocation between *EWSR1* and a variety of *ETS* partner transcription factors[5]. These gene fusions act as oncogenic transcription factors that trigger transcriptomic and epigenetic disregulations that explain tumors' biology[6,7]. In contrast to EWS and RMS,

osteosarcoma shows an extremely complex and unstable genome but without a remarkable repetitive pattern[8]. In most clinical settings, sarcomas involving translocations are detected by fluorescence in situ hybridization (FISH) and reverse transcriptase polymerase chain reaction (RT-PCR). Translocations are used by clinicians mostly as diagnostic markers[9]. However, the resulting chimeric proteins of these translocations are not easily druggable and hinder the development of inhibitors. **Table 1** shows the most frequent fusion transcripts in pediatric sarcomas.

Both STS and BS display a highly aggressive behavior. During the last decades, addition of systemic chemotherapy has improved outcome of localized tumors resulting in the survival of 2 out of three patients. However, metastasic and relapsed sarcomas still have very poor survival rates. Despite the knowledge gained in cancer biology, aetiology and in the implementation of novel diagnostic techniques and omics, scarce improvement has been observed in advanced stage STS and BS.

During the last years, precision and quality criteria for the diagnosis of pediatric cancers including sarcomas, has experienced an increased demand. New techniques have been introduced that complement pathological diagnosis including immunochemistry, FISH, RT-PCR and next-generation sequencing (NGS). These demands have been gradually assumed by clinicians, pathologists, geneticists and molecular biologists in tertiary reference hospitals. In addition, precision medicine programs have been developed in order to expand our knowledge of tumor biology and defeat cancer with more precise pharmacological targets.[10,11,12,13] We present the results in pediatric sarcomas from the Precision Medicine program for children and adolescents with solid tumors in relapse/progression carried out at a national reference centre for pediatric sarcomas. This program has received samples from collaborative centres, providing a national perspective[14]. Since September 2019, these studies are routinely carried out at diagnosis in every pediatric sarcoma.

Materials And Methods

Study subjects

A total of 70 sarcoma samples from pediatric patients treated at a reference institution for pediatric sarcomas or at other Spanish center from February 2015 to March 2020 were included. Thirty patients were analyzed at diagnosis and forty patients were studied at relapse or refractory disease.

The program was approved by the Ethics committee of the center. Parents signed the informed consent and were informed about the possibility of finding germline mutations and accepting or refusing to be informed. Consent was also required when performing NGS studies at diagnosis. Every procedure was performed according to the Declaration of Helsinki.

Study samples

Fresh tumor samples were requested. Paraffined-embedded tumors and/or pretreatment biopsies were only used if fresh samples were unavailable. Peripheral blood samples were simultaneously collected in 45 cases. All tumor samples were reviewed by a board-certified pathologist to confirm histology and

estimate tumor cell content. Immunochemistry techniques (p-AKT, PDL1, p-EGFR, c-KIT, PTEN, Her2neu, p53) and FISH (NTRK1 / 3, ALK, BRAF) were also performed. Only samples with > 30% tumor cell content were considered for further genomic testing, the rest were excluded from the study. The selected tumor material and peripheral blood samples were sent to a biobank for DNA extraction and subsequently to the laboratory for sequencing analysis. In some cases, based on previous literature and according to the sequencing results obtained for each tumor type, studies were completed with SNP array analysis.

DNA extraction

DNA extraction from tumor and blood samples was carried out using the QIAamp® DNA Investigator kit (QIAGEN® ref.56504) or QIAamp® DNA Mini Kit (QIAGEN® ref. 51, 304, respectively, following manufacturer instructions. The concentration and absorbance ratios were measured with NanoDrop 2000®13.

RNA extraction and cDNA generation

RNA was extracted with the RecoverAll™ Total Nucleic Acid Isolation Kit following manufacturer's protocol. Total RNA was quantified with the with the Qubit™ RNA HS Assay Kit (ThermoFIsher Scientific), and cDNA was obtained with the SuperScript™ IV VILO™ Master Mix.

Sequencing studies, data interpretation and variant calling

Commercial and customized NGS panels that included the consensus gene list and that produced an average coverage of 1000X and homogeneity with a minimum of 85% were used for the analysis of relapse or refractory patients: Ion Ampliseq Cancer Hotspot Panel v2 (Thermo Fisher Scientific), Human Comprehensive® Cancer Panel (Qiagen©), Pediatric-OncoPanelDx® (Imegen) and Onconano Gene Panel (Pediatric Oncology Group-IIsLaFe). For analysis of newly diagnosed samples, the Oncomine Childhood Research Assay® was used (Ref: A36485).

Gene panels included at least the following: *ABL1, AKT1, ALK, BRAF, CDKN2A, CSF1R, CTNNB1, EGFR, ERBB2, ERBB4, EZH2, FGFR1, FGFR2, FGFR3, FLT3, GNA11, GNAQ, HRAS, IDH1, IDH2, JAK2, JAK3, KIT, KRAS, MET, MPL, NRAS, PDGRFA, PIK3CA, PTEN, PTPN11, RB1, RET, SMARCB1, SMO*, and *TP53*.

For NGS data analysis, variant calling was based on the genome version GRCh37 (hg19). Genetic variants detected in both, blood and paired tumor samples were classified as germline variants, whereas variants detected exclusively in tumors were categorized as somatic variants.

Variant annotation was carried out applying an algorithm of filters in order to discard non clinically relevant variants: those with an allelic frequency < 5%, changes in non-coding regions (excluding those variants in exon splicing sites +/− 10 nucleotides), synonymous variants (excluding those coding variants nearby splicing sites +/− 4 positions), variants with high frequency in the general population (MAF > 0.01), polymorphic changes (SNPs) without clinical relevance found in healthy population or described as benign by several sources or our genomic database. The remaining variants were classified according to international recommendations as pathogenic, likely pathogenic, benign, likely benign or of uncertain

significance[13] based on literature or disease databases (ClinVar, COSMIC, HGMD, St Jude PeCan or CiVIC).

Pathogenic or likely pathogenic variants were reviewed and approved by the pediatric molecular tumor board (PMBT) committee, and further confirmed using direct Sanger sequencing. Actionable variant was referred as a genomic change that suggests an alteration with biological activity that could be targeted with a specific therapy already used in vivo. Targeted therapies were preferentially recommended to be administered within clinical trials but also as compassionate use basis if trials weren't available. Median time between biopsy/surgery and molecular tumour board recommendation was 5 weeks.

Pediatric molecular tumor board discussion

The PMTB was created in November 2014 and composed by pediatric oncologists, pharmacologists, geneticists, pathologists, molecular biologists and bioinformatics. The PMBT established the consensus gene panel for the NGS analysis. After the completion of pathological and genomic studies, results were discussed in periodical meetings in the PMTB and a final report was transferred to the corresponding physician. The workflow was based on previous pilot studies[14,15].

Results

Clinical characteristics

A total of 70 pediatric and adolescent patients with STS and BS from 10 Spanish cooperating sites were included in a 5-year period from February 2015 to March 2020. Patients' median age at study entry was 11.5 years with a range of 1-18 years. Forty-one per cent of the patients were female and 59% were male. Distribution of tumor type is shown in **Figure 1**. The most frequent tumors were EWS (n=22), RMS (n=16) and osteosarcoma (n=13). Thirty patients were studied at diagnosis (43%), 22 patients at first relapse (31%), 15 patients at second or successive recurrences (21%) and 3 patients when found to be refractory to first line treatment (4%). Somatic variants described as pathogenic or likely pathogenic using international system classifications[15] were reported. The main characteristics of the selected patients are detailed in **Table 2**.

NGS results

Twenty-two out of 70 patients (31%) had at least one pathogenic or probably pathogenic alteration identified by NGS as with a mean of 1,4 mutations per patient. Most of the cases had one unique mutation. A total of 30 different pathogenic or likely pathogenic mutations in 18 different genes were detected amongst the 22 patients. Mutations were detected in relapsed or refractory sarcomas (57%) and also at first diagnosis (43%).

Diagnostic sarcoma fusion genes detected by FISH or RT-PCR were only used for diagnosis but were not considered for precision medicine recommendations as no targeted treatments are available for these alterations to date. Overall, *TP53* was the most frequently affected gene (27%) and preferentially

identified in EWS, RMS and angiosarcoma. Three embryonal rhabdomyosarcomas harbored alterations in *FGFR4* whilst the two aggressive fibromatosis and an embryonal RMS had *CTNNB1* mutations. Identified gene and variant alterations are shown in **Table 3**. Including information obtained by complementary techniques (immunochemistry and FISH) up to 27 patients had an identified alteration (39% of the cases). A summary of the molecular alterations spotted in these 27 patients is shown in **Figure 2.**

Clinical translation

After discussion of the biological results in the PMTB, 18 actionable variants (26%) were identified and formal recommendations were submitted to the respective physicians. RMS was the tumor in which more actionable variants were observed (39%), particularly embryonal histology (28%). Two osteosarcoma patients presented actionable alterations. Despite the number of EWS cases included in the study (22), only one patient had an actionable variant. This result points out the difficulties to implement a precision medicine strategy in EWS tumors.

Six patients out of the whole cohort received targeted treatment (9%), observing clinical benefit in five of them (78%):

A thirteen-year-old female with a radio-induced abdominal malignant nerve sheath tumor, 10 years after neuroblastoma treatment was found to have a mutation in *ATM.* After radical surgery and standard chemotherapy, she underwent disease progression. Targeted treatment with PARP inhibitor olaparib and temozolamide was administered, resulting in disease stabilization during one month with a clear disease control. She received treatment during two months before a subsequent progression.

A twelve-year-old female with malignant perivascular epithelioid cell kidney tumor with lung metastasis was found to have positive p-AKT with immunochemistry and targeted treatment with sirolimus and sorafenib was initiated after observing no response to classic sarcoma chemotherapy. A slight response was observed in tumor size and the disease was stabilized according to RECIST 1.1 criteria for a 5-month period. This achievement had not been possible with the previous schedules administered. On the third place, a nine-year old female with first local and metastasic osteosarcoma relapse with positive mTOR immunochemistry was treated with an oral mTOR inhibitor during a two-month period after failure of standard treatments. Unfortunately, progression was observed after the third month.

Another twelve-year-old male affected by a mediastinal myofibroblastic inflammatory tumor with ALK translocation detected by FISH. Disease progression was observed after standard chemotherapy (IVA regime) and surgery. Targeted treatment with *ALK* inhibitor ceritinib was started and a very good partial response was observed. Finally, a thirteen-year-old female with a stomach GIST with positive c-KIT diagnosis (CD-117) by immunohistochemistry is currently receiving imatinib after radical surgery and has achieved complete response.

Future treatment options were available in 12 patients (17%) that are at the moment in complete response or receiving other standard treatments. Altogether, implementing NGS with complementary

diagnostic techniques such as immunohistochemistry and FISH in a precision medicine approach for targeted treatment of sarcomas, a disease control rate of 23% would potentially be achieved. The summary of the recommendations and clinical responses are shown in **Table 4.**

Discussion

Genetic variation is one of the main characteristics of pediatric sarcomas. This is mostly explained because despite being originated from a mesenchymal cell, they constitute different histologic entities with different genomic landscapes that explain their unequal behaviours. Beside pathology, chromosomal segmental aberrations,[16] changes in ploidy and specific gene alterations are routinely used in order to guide intensity of treatment in pediatric oncology protocols.

It is worth noting important differences spotted when comparing adult with pediatric NGS studies in sarcomas.[17] Epidemiologically, sarcomas represent less than 1% of all solid malignant cancers in adult population while they represent 20% of all pediatric solid malignant cancers. Therefore, the first main difference lies in the fact that the magnitude of the problem is proportionally much higher in pediatric population. Furthermore, adult type cancers such as epithelial neoplasms arise after accumulation of multiple sequential mutations directly linked to environmental exposures, and arise within differentiated adult tissues[18,19]. Mesenchymal tumors, such as sarcomas appear both in adult and pediatric population. However, specific histologic subtypes and clinical progression are age-dependent, suggesting differential pathogenetics and underlying molecular mechanisms for tumor initiation and clinical behavior in the different age subgroups[18].

In this study, we found that the overall mutational load in our cohort was relatively low when compared to adult studies. This might be explained by the fact that adult sarcomas are mostly driven by mutagenic exposure from environmental factors, whereas most of pediatric cancers contain a relatively small number of mutations[20] and frequently display unique gene rearrangements. Although this restricts the targeted treatment to available drugs, it also makes them attractive candidates for drug discovery[15].

In order to improve outcome, international efforts amongst cooperative groups have been carried out developing genomic precision medicine programs. These programs aim to bring NGS approaches into the clinical practice and require the identification of patients that might benefit from targeted therapies. Once these targets are identified, in pediatric population it is important to communicate these results, as well as possible toxicities observed by compassionate use basis as dosing is more complex when compared to adult population. Hence, the importance of promoting pediatric phase I clinical trials in order to titrate infant dosing.

In this study, we conclude that the most frequent somatic mutation observed in pediatric sarcomas occurs in TP53 (27% of the pathogenic mutations detected by NGS). This information correlates with adult sarcoma cohorts such as the study presented by *Groisberg et al.*[21] Xiaosheng et al[22] compared overall survival (OS) time between *TP53*-mutated and *TP53*-wildtype cancers in 20 adult cancer types.

They reported that patients with *TP53* mutations had lower survival compared with those without *TP53* mutations in colon, lung and pancreas adenocarcinoma, acute myeloid leukemia and other epithelial cancers. In pediatric oncology, the clinical significance of somatic *TP53* mutations remains unrecognized and no routine testing or therapy intensification is considered. Recent studies suggest that mutation in *TP53* in localized EWS is not a reliable prognostic marker[23]. In order to target *TP53*, small molecules that reactivate mutant p53 by restoring wild-type conformation have been identified by various approaches. *APR-246* alone is currently being tested in prostate or ovarian cancers or in combination with azacitidine in myeloid malignancies in adult phase I-II trials. No studies are currently recruiting pediatric population.

Mutations in Fibroblast Growth Factor Receptor 4 (FGFR4) have also been described in pediatric sarcomas, most outstandingly in RMS. Higher *FGFR4* expression in RMS has been associated with advanced-stage cancer and poor survival[24]. *FGFR4* pathogenic mutations appear in 33% of the embryonal RMS studied in our cohort and all of them received a targeted recommendation therapy. *FGFR4* codifies for a cell surface tyrosine kinase (TK) receptor that is involved in normal myogenesis and muscle regeneration. It has been reported that human embryonal RMS cells have increased FGFR4 mRNA expression compared to normal human myoblasts, and FGFR4 pathway blockade decreases proliferation.[25] In fact, over-expression and mutational activation of *FGFR4* has been reported in RMS, promoting tumor progression. *FGFR4* signaling is also a common mechanism of oncogenesis in fusion positive RMS (usually alveolar subtype)[25].

Alterations in *FGFR4* are clinically relevant because they are actionable targets in patients with RMS. New generation of multikinase inhibitors are under current development such as ponatinib (AP-24534), an orally administered TK inhibitor that was initially developed as an inhibitor for *BCL-ABL*. Ponatinib recently received FDA approval for the treatment of adult patients with Philadelphia chromosome positive acute lymphoblastic leukemia and chronic myeloid leukemia resistant to other TK inhibitors. Inhibition profile of ponatinib includes other TK such as c-KIT, PDGFR, FLT3, SRC and FGFR[26]. Moreover, inhibition of FGFR family members with ponatinib has been demonstrated in preclinical models with bladder cancer, endometrial cancer, breast, lung and colon cancer. *Samuel Q. Li et al*[26] tested a panel of RMS cell lines over-expressing FGFR4, all of them exhibiting sensitivity to five different TK inhibitors including ponatinib, cediranib, nintedanib, dovitinib and danusertib. They observed that ponatinib resulted to be the most powerful FGFR4 inhibitor, inhibiting both, mutated and wild-type FGFR4 cell growth. It also inhibited tumor development expressing FGFR4 in vivo[26]. Currently, ponatinib is being tested in clinical trials including pediatric patients (NCT03934372)[25,27]. Erdafitinib is also being tested in a phase II trial for tumors with FGFR mutations. (NCT03210714).

The CTNNB1 gene provides instructions to form the protein beta-catenin. The relationship between the Wnt/beta-catetin signaling pathway and desmoid-type fibromatosis (DTF) has been widely studied and it has been reported that the vast majority of DTF tumors (up to 85%) harbor a mutation in exon 3 of the *CTNNB1* gene (beta-catetenin)[28]. These mutations lead to an abnormally stable beta-catenin protein that

is more resistant to proteolytic degradation and accumulates within the cells. Excess of beta-catenin promotes an uncontrolled proliferation of cells, allowing the formation of DTF[29].

Therapeutic options targeting Wnt/betacatenin signaling pathway are limited and have not been tested in pediatric population. Accumulation of beta-catenin in the nucleus triggers transcription of Wnt-specific genes responsible for the control of cell fate decisions. The development of drugs targeting mutated or altered beta-catenin signaling, or its interaction with CBP, TCF, GSK3β or APC (which are essential to complete its function) has been difficult due to the toxicity of the new compounds. Several of them are currently in Phase 1 clinical trials, such as the PRI-724 molecule (NCT01302405, NCT02413853, NCT01764477, and NCT01606579) that prevents the interaction of beta-catenin with CBP. Despite these and other approaches, there are no clinical trials available for pediatric patients with Wnt/beta-catenin inhibitors.[30] All DTF studied in our cohort harbored mutations in *CTNNB1*.

In the study, a patient with malignant nerve sheath tumor and ATM mutation was treated with PARP inhibitors in combination with olaparib. The ataxia telangiectasia gene (*ATM*), localized in 11q22-q23, plays an important role in maintaining genomic integrity. It regulates the double-strand DNA breaks repair and activates different checkpoints in the cell cycle. *ATM* is associated with some types of leukemia and lymphoma and it has also been described in neuroblastoma with 11q deletion. Poly ADP-ribose polymerase (PARP) is a protein that signals DNA damage and contributes towards DNA repair[31]. PARP catalyzes the addition of ADP-ribose to DNA, helicases, topoisomerases and histones. It also has a critical role in transcription, cellular replication, gene regulation, differentiation, spindle maintenance and protein degradation. PARP inhibition produces persistent single strand DNA breaks leading to double strand DNA breaks and finally produces DNA damage leading to apoptosis and cell cycle arrest. Preclinical studies show that *ATM* mutated neuroblastoma cells also succumb to apoptosis when treated with PARP inhibitors and neuroblastomas with 11q deletion are extremely sensitive to conventional chemotherapy combined with PARP inhibitors. The patient in the study managed a short period of stable disease but progressed rapidly afterwards[31]. Other mutations considered as uncertainly significant in *ATM* have been detected but no recommendations were issued because no previous clinical evidence was found. Currently, early phase trials with PARP inhibitors are recruiting pediatric patients with diverse malignancies.

Recent studies in RMS have revealed recurrent mutations in the RAS pathway, particularly affecting NRAS. *Dolghik et al*[32] demonstrated that *PIK3CA* played a critical role in the activation of the PI3K/AKT/mTOR pathway in *NRAS* mutant RMS. They noted that NRAS-mutated RMS cells particularly relied on *PIK3CA* to prevent cell death upon *NRAS* silencing or *MEK* inhibition. Their data showed that specific *PIK3CA* knockdown was sufficient to cooperatively trigger cell death together with pharmacological *MEK* inhibition. In addition, pharmacological inhibitors of *MEK* or *NRAS* knockdown synergize with the *PIK3CA* specific inhibitor BYL719 to trigger cell death in *NRAS*-mutated RMS cells. All this data supports the rationale for the combination of *MEK* and *PIK3CA* specific inhibitors in *NRAS* mutated RMS. This recommendation is a future option for one of the patients studied in our cohort.

In this study, a patient diagnosed with **c**-KIT positive (CD-117) GIST was treated with imatinib and so far, has maintained complete response after surgery. Another patient with ALK + myofibroblastic inflammatory tumor received treatment with ceritinib obtaining a partial response. Both of these rare sarcomas have a classical alteration that has been widely reported before.

In conclusion, we have observed that the incorporation of NGS results together with ancillary studies into pediatric sarcoma clinical practice is feasible and allows personalized treatments with acceptable disease control rates in the relapse setting. At the moment, as the integration NGS as a routine diagnostic technique has been limited this is difficult to estimate, although the situation is changing and sequencing studies are gradually becoming widespread[33,34,35]. Further investigations are required to confirm this hypothesis.

In this study, up to 23% of the patients would obtain clinical benefit by implementing this precision medicine approach complementing routine diagnostic techniques. Although the understanding of pediatric sarcomas' biology has improved in a relatively short period of time, outcomes in high-risk tumors remain poor and regarding new therapeutic strategies, very few advances have been highlighted. This emphasizes that strong, international efforts are still required in order to improve implementation of new diagnostic techniques, impulse pediatric drug development and access to clinical trials in childhood. Finally, we would like to stress the importance of treating childhood, adolescent and young adult sarcomas and other types of cancers in specialized units, with all the available expertise and distinct requirements involving this particular population.

Declarations

Ethics approval and consent to participate

Informed consent for all the patients was obtained from the parents or legal guardians. All study actions have been done under the appropriate ethics code and the study has been approved by the Ethics and Investigation Committee Hospital U i P La Fe (CEIm). The study was performed according to the Declaration of Helsinki.

Consent for publication

Consent for publication has been obtained from all patients or, if patients are under 18, from a parent and/or legal guardian.

Availability of data and materials

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Competing Interests

The authors declare no competing financial interests and no other non-financial competing interests.

Funding

Funding has been obtained from private micro-donations, and these from patient associations.

Authors' contributions

All authors read, corrected and approved the final manuscript. All the authors (AJR, PG, PB, VS, YY, BJ, MS, MLL, JF, VC, AC) have contributed in an essential way obtaining data, joint analysis and obtaining conclusions in the text.

Acknowledgements

*Désirée Ramal has contributed in an essential way in the study development and management of the data from the project.

*Hospital U i P La Fe is a full member of the "European Reference Network for pediatric Oncology (ERN-PAEDCAN)" and national reference centre in pediatric sarcomas (CSUR).

*We would like to thank all the centres that contributed in the study:

- Hospital General Universitario de Alicante

- Hospital Clínic universitari de Valencia

- Hospital Universitari Son Espases

- Hospital San Rafael

- Hospital Clínico Universitario Virgen de la Arrixaca

- Hospital Universitario Virgen del Rocío

- Hospital Universitario Virgen de las Nieves.

- Complejo Hospitalario Universitario de Vigo

- Hospital Universitario de Donostia

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Tables

Table 1. Main fusion transcripts in pediatric sarcomas: Chromosomal translocation, gene transcripts and expected frequences. RMS: rhabdomyosarcoma; STS: soft-tissue sarcoma.

DIAGNOSIS	TRANSLOCATION	FUSION	FREQUENCY
Alveolar RMS	t(2;13)(q35;q14) t(1;13)(p36;q14)	PAX3 / FOXO1 PAX7 / FOXO1	60% 20%
Ewing sarcoma	t(11;22)(q24;q12) t(21;22)(q22;q12) t(7;22)(p22;q12) t(2;22)(q35;q12) t(16;21)(p11;q22)	EWSR1 / FLI1 EWSR1 / ERG EWSR1 / ETV1 EWSR1 / FEV FUS / ERG	85% 10% <1% <1% <1%
Desmoplastic small round cell tumor	t(11;22)(p13;q12.2)	EWRS1 / WT1	>95 %
Infantile fibrosarcoma	t(12;15)(p13.2;q25.3)	ETV6 / NTRK3	70 %
Synovial sarcoma	t(X;18)(p11.2;q11.2-11.23) t(X;18)(p11.2;q11.2-11.23) t(X;18)(p11.2;q11.2-11.23)	SS18 / SSX1 SS18 / SSX2 SS18 / SSX4	64% 35% 1%
Clear cell STS	t(12;22)(q13;q12)	EWSR1 / ATF1	>90%

Table 2. Clinical characteristics of the studied population (n=70). STS: soft-tissue sarcoma.

VARIABLE	N (n=70)
DIAGNOSIS	
Ewing Sarcoma	22
Rhabdomyosarcoma	16
Osteosarcoma	13
Desmoplastic small round cell tumor	3
Infantile fibrosarcoma	3
Undifferentiated sarcoma	3
Desmoid-type fibromatosis	2
Malignant nerve sheath tumor	2
Clear cell STS	2
Angiosarcoma	1
Malignant Perivascular Epithelioid Cell Neoplasm	1
Gastrointestinal stromal tumor	1
Myofibroblastic inflammatory tumor	1
AGE	
0-4	6
5-8	16
9-12	20
13-18	28
SEX	
Female	29
Male	41
CLINICAL STATUS	
Diagnosis	30
First relapse	22
Second or next relapses	15
Refractory	3

Table 3. Tumor sequencing results: Mutated gene, variant, sarcoma subtype and clinical status. DSRCT: desmoplastic small round cell tumor; RMS: rhabdomyosarcoma; EWS: Ewing sarcoma; DTF: desmoid-type fibromatosis; MPNST: malignant peripheral nerve sheath tumor.

GENE	VARIANT	TUMOR TYPE	CLINICAL STATUS
TP53	c.1040C>T(p.A347V)	DSRCT	Relapse
	c.559G>C(p.G187R)	Embryonal RMS	Diagnosis
	c.906_907delCCinsTT (p.R303*)	Angiosarcoma	Diagnosis
	c.742C>T (p.R248W)	EWS	Diagnosis
	c.404G>T (p.C135F)	EWS	Relapse
	c.817C>T (p.R272C)	Alveolar RMS	Refractory
	c.448A>G (p.Y163C)	EWS	Relapse
	c.614A>G(p.Y205C)	EWS	Relapse
FGFR4	c.1648G>A(p.V550M)	Embryonal RMS	Relapse
	c.1648 G>C (p.V550L)	Embryonal RMS	Diagnosis
	c.1648 G>C(p.V550L)	Embryonal RMS	Diagnosis
CTNNB1	c.134C>T(p.S45F)	DTF	Diagnosis
	c.134C>T(p.S45F)	DTF	Relapse
	c.133_134delTCinsCT (p.S45L)	Embryonal RMS	Diagnosis
GENE	**VARIANT**	**TUMOR TYPE**	**CLINICAL STATUS**
SMAD4	c.302G>A(p.W101*)	Alveolar RMS	Relapse
	c-370G>A(p.D124N)	Alveolar RMS	Relapse
ATM	c.7032G>A(p.W2344*)	MPNST	Diagnosis (secondary tumor)
NRAS	c.176C>T (p.A59V)	Alveolar RMS	Relapse
CIC	c5939_5943del (p.G1980Vfs*78)	Clear cell renal sarcoma	Relapse
FBXW7	c.1394G>A (p.R465H)	Embryonal RMS	Diagnosis
RB1	c. 361 C>T (p.Q121*)	Osteosarcoma	Relapse
AKT1	c.138C>A(p.D46E)	MPNST	Relapse
JAK3	c.2164G>A(p.V722I)	EWS	Diagnosis
PI3K	c.1624G>A(p.E542K)	Embryonal RMS	Relapse
SMARCB1	c.1135>A (p.A379T)	Alveolar RMS	Relapse
MLH1	c.1138G>A(p.A380T)	Alveolar RMS	Relapse
MTOR	c.6644C>T(p.S2215F)	Angiosarcoma	Diagnosis
TSC2	c.5158C>T(p.R1720W)	Embryonal RMS	Relapse
SMARCA4	c.1135>A (p.A379T)	DSRCT	Diagnosis
NF1	c.2087G>A (p.W696*)	EWS	Diagnosis

Table 4. Clinical translation: Actionable variants detected, molecular tumor board recommendations, targeted treatments administered and results observed. EWS: Ewing sarcoma; IQ: immunochemistry; RMS: rhabdomyosarcoma; DTF: desmoid-type fibromatosis; MPNST: malignant peripheral nerve sheath tumor; LOH: loss of heterozygosity; CNV: copy number variation; MIT: myofibroblastic inflammatory tumor; GIST: gastrointestinal stromal tumor; MPECN: malignant perivascular epithelioid cell neoplasm.

TUMOR HISTOLOGY	ACTIONABLE VARIANT	OTHER BIOLOGIC INFORMATION	COMMITTEE RECOMEN-DATION	TREATMENT ADMINISTERED	MAXIMUM RESPONSE
EWS	*TP53* c.742C>T (p.R248W)	*PDL-1* + (IQ 5%)	*PRIMA-1* / *PD-L1* inhibitors	No (future option)	-
Embryonal RMS	*FGFR4* c.1648 G>C (p.V550L) *FBXW7* c.1394G>A (p.R465H)	-	Ponatinib / Erdafitinib	No (future option)	-
Embryonal RMS	*TP53* c.559G>C (p.G187R) in germline	*P-AKT* + (IQ 50%)	*mTOR* inhibitor. Li-Fraumeni follow-up	No (future option)	-
Embryonal RMS	*TSC2* c.5158C>T (p.R1720W) *FGFR4* c.1648G>A (p.V550M)	*mTOR* + (IQ 100% cytoplasm)	*mTOR* inhibitor	No (future option)	-
Embryonal RMS	*PI3K* c.1624G>A (p.E542K)	-	*mTOR* inhibitor	No (future option)	-
Embryonal RMS	*FGFR4* c.1648 G>C (p.V550L) *CTNNB1* c.133_134delT CinsCT (p.S45L)	-	Ponatinib / Erdafitinib	No (future option)	-
Alveolar RMS	*SMAD4* c.302G>A (p.W101*) *SMAD4* c-370G>A (p.D124N) NRAS c.176C>T (p.A59V)	-	Palbociclib + Venetoclax	Yes (<1 month)	PD
Alveolar RMS	*TP53* c.817C>T (p.R273C)	-	*PRIMA-1*	No (future option)	-
Angiosarcoma	*TP53* *c.906_907del CCins7TT (p.R303*)* *MTOR* c.6644C>T (p.S2215F)	*P-AKT* + (IQ 60% membrane and cytoplasm) Patient with Xerodermapigm entosum	*mTOR* inhibitor	No (future option)	-
DTF	*CTNNB1* c.134C>T (p.S45F)	IQ betacatenin +	Beta-catenin inhibitor	No (future option)	-
DTF	*CTNNB1* c.134C>T (p.S45F)	IQ betacatenin +	Beta-catenin inhibitor	No (future option)	-
MPNST	*ATM* c.7032G>A (p.W2344*)	11q deletion	*PARP* inhibitor	Yes (2 months)	SD
Osteosarcoma	*RB1* c. 361 C>T (p.Q121*)	Gain chromosomes: +14,+20,+21, Segmental imbalances: 2p, 17q. LOH 3, 16. *PD-L1* + (IQ 20%)	*PD-L1* inhibitors	No (medical decision)	-
Osteosarcoma	-	*mTOR* + (IQ 60% cytoplasm)	*mTOR* inhibitor	Yes (2 months)	SD
Undifferentiated sarcoma	-	CNV: Deletion in genes *ARID1A, MTOR, NRAS, SDHB*	Tazemetostat / Vorinostat	No (future option)	-
MIT	-	FISH: *ALK* +	Ceritinib	Yes (3 months)	PR
GIST	-	IQ: C-KIT+ (CD117)	Imatinib	Yes (20 months)	CR
MPECN	-	*P-AKT* + (IQ 100%)	Sirolimus + Sorafenib	Yes (5 months)	SD

Figures

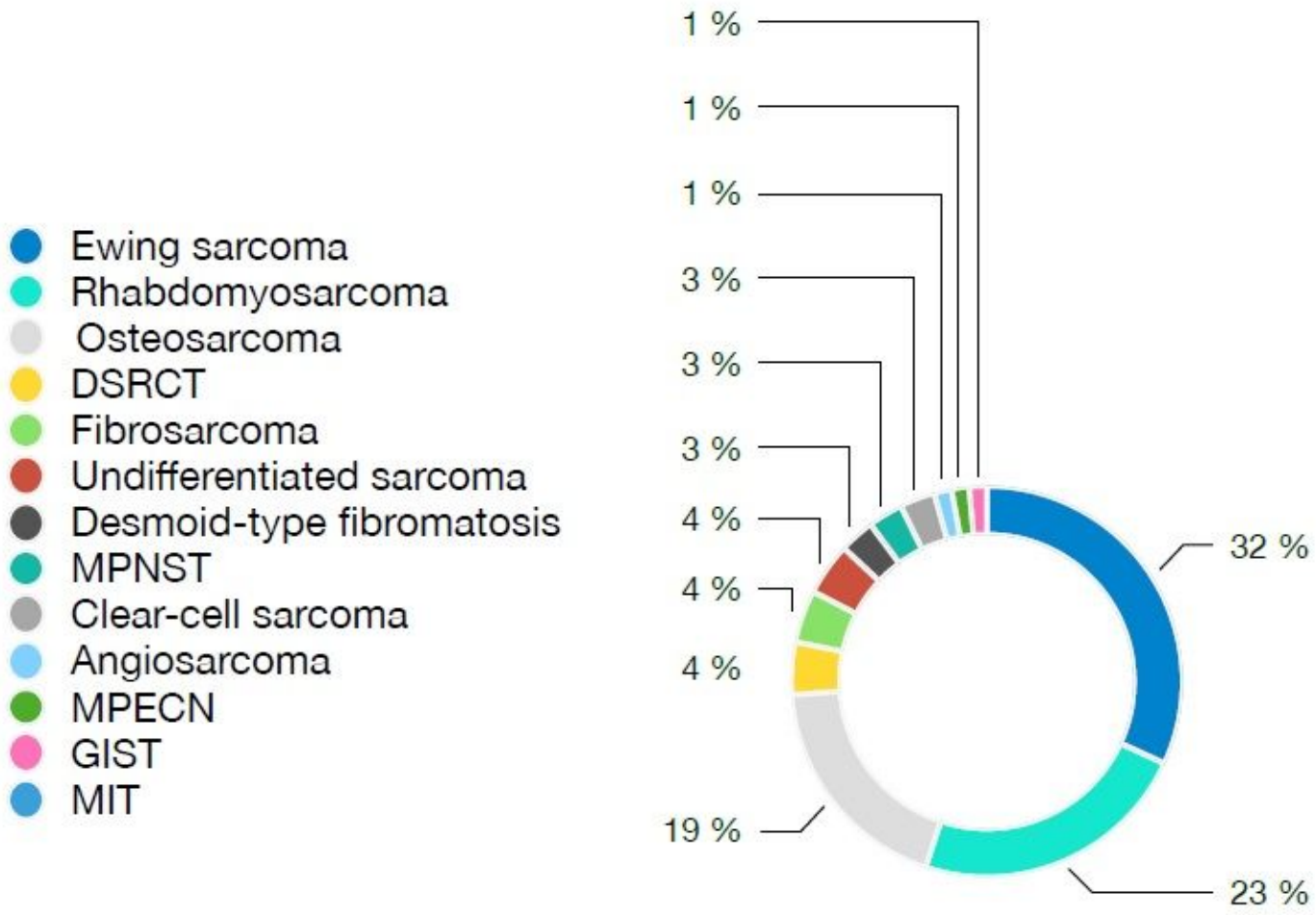

Figure 1

Distribution of sarcoma type amongst reported cases. DSRCT: desmoplastic small round cell tu-mor; MPNST: malignant peripheral nerve sheath tumor; MPECN: malignant perivascular epithe-loid cell neoplasm; GIST: gastrointestinal stromal tumor; IMT: myofibroblastic inflammatory tu-mor.

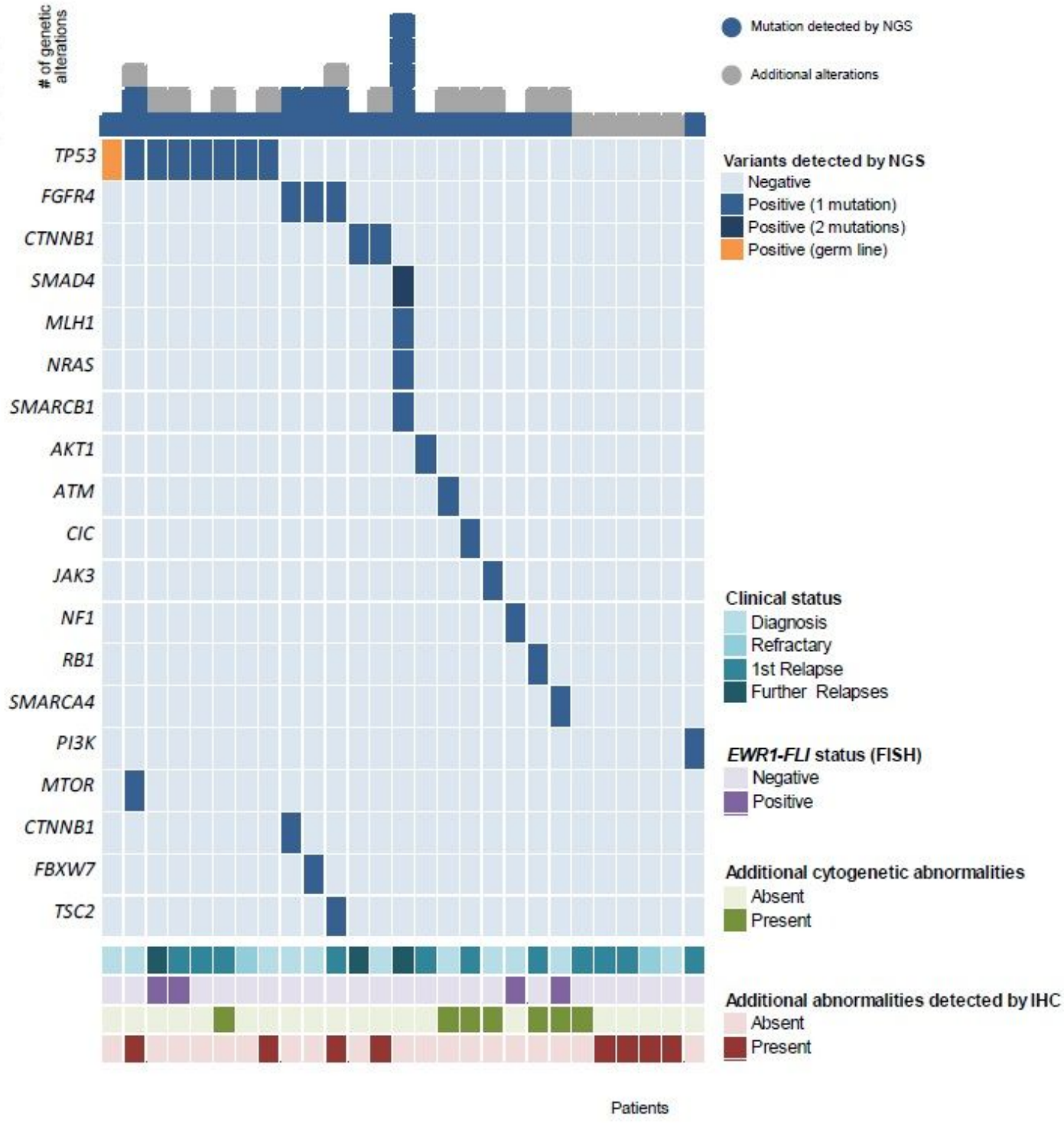

Figure 2

Molecular alterations observed in the 27 patients (each column represents one patient). IHC: immunochemistry.