

Next-generation personalised medicine for high-risk paediatric cancer patients – The INFORM pilot study

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1. Introduction

Current treatment of childhood malignancies using modern multimodal therapies results in a 5-year overall survival (OS) of around 80% [1]. Nevertheless, certain high-risk entities remain a substantial clinical problem. Cure rates for these tumours of less than 20% after recurrence imply an urgent need for innovative treatment strategies [2–13]. Whilst progress from a clinical perspective has slowed in recent years, major advances have been made in understanding cancer biology. Multiple pipelines are now being developed to translate this knowledge and to bring next-generation sequencing-based approaches into clinical application. Compared with adult cancers, which can be driven by, e.g. chronic mutagenic exposure from environmental factors, most paediatric cancers carry relatively few mutations [14,15]. This makes them attractive candidates for identifying

therapeutic targets, since distinguishing driver mutations from background alterations should be comparatively easier. Until recently, the genetic underpinnings of many paediatric cancers were largely unknown, with the exception of hereditary predisposition in 5–10% of cases [16]. Large-scale sequencing efforts in the framework of the International Cancer Genome Consortium (ICGC), the Paediatric Cancer Genome Project and other initiatives (<https://icgc.org>; <http://explore.pediatriccancergenomeproject.org>) [17–19] have yielded a plethora of potential new therapeutic options. Inspired by these opportunities, 11 study groups of the Society for Pediatric Oncology and Hematology (GPOH) as well as the German Cancer Consortium (DKTK) established the Individualized Therapy for Relapsed Malignancies in Childhood (INFORM) project to enable access to cutting-edge molecular diagnostics for high-risk paediatric oncology patients across Germany.

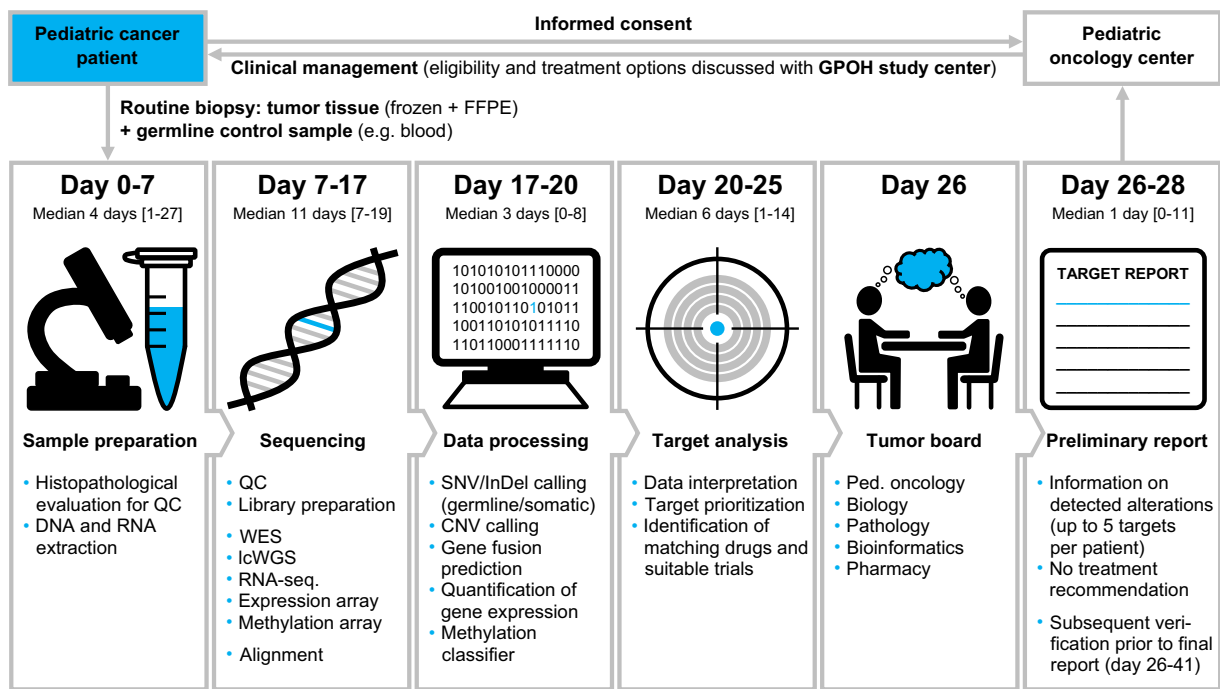


Fig. 1. Schematic overview of the INFORM workflow and timelines. The mean time intervals are given on top of the boxes, followed by the median time needed for each step and the range with minimal and maximal values (indicated by square brackets). CNV, copy number variants; FFPE, formalin-fixed paraffin embedded; FISH, fluorescence in situ hybridisation; GPOH, Society for Pediatric Oncology and Hematology; InDel, small insertion or deletion; INFORM, Individualized Therapy for Relapsed Malignancies in Childhood; lcWGS, low-coverage whole-genome sequencing; QC, quality control; RNA-seq., RNA sequencing; SNV, single-nucleotide variants; WES, whole-exome sequencing.

The comprehensive analysis conducted within this setting provides a molecular profile of each individual tumour, which is assessed for clinical relevance by an expert panel. The whole process from sample delivery to molecular target report is intended to take less than 4 weeks.

We enrolled 57 patients in a pilot study in order to identify and overcome challenges of such an approach. The goals were to (i) Establish logistics for real-time molecular analysis and target identification and (ii) assess relapsed high-risk paediatric malignancies with respect to patterns of potential drug targets. Thus, we aimed to provide the basis for future biomarker-driven, cross-entropy trials.

In addition to demonstrating the feasibility of the program as a whole, this pilot project provided new biological information and produced results which could be immediately translated into therapeutic application in several patients. The key findings, presented here, highlight important considerations for such enterprises in future.

2. Methods

2.1. Study design and participants

Eligible patients included children, adolescents and young adults aged 1–40 years with refractory/relapsed/

progressive oncological disease (with initial treatment according to a GPOH protocol) as well as specific primary indications. Detailed inclusion criteria are outlined in the [Supplementary Methods](#) and [Supplementary Table 1](#). Written informed consent covering sequencing analysis of tumour and germline, exchange of clinical and molecular data between the GPOH study groups and the coordinating center in Heidelberg as well as scientific evaluation of molecular and clinical results was obtained at the local center. Patients and parents could decide whether they wanted to be informed about cancer-related germline alterations. Ethics committee approval for conducting the study, use of consent forms and scientific evaluation of the data were obtained from Heidelberg University Hospital's review board.

2.2. The INFORM workflow

An overview of the project workflow is outlined in [Fig. 1](#) and described in the [Supplementary Methods](#). Briefly, fresh frozen tumour material from the current disease manifestation and matching non-neoplastic material were subjected to quality control and analyte extraction prior to molecular analysis. Tumour content was required to be >40% on histological assessment. Bioinformatic processing is outlined below and in the [Supplementary data](#). An expert panel prioritised

potential targets for reporting to the treating physician, who could then use the information for clinical decision making on an individual basis.

For germline analysis, we closely collaborated with our local human geneticists, who were consulted whenever we detected variants in cancer predisposition genes that were not listed as a polymorphism (<http://www.ncbi.nlm.nih.gov/SNP> build 135, <http://www.1000genomes.org>, <http://exac.broadinstitute.org/>) and were suspected to be tumour relevant (Supplementary Table 2).

Table 1
Characteristics of enrolled patients (n = 57).

Characteristic	No.	%
Age (years)		
Median	13	
Range	1–40	
Sex		
Male	30	53
Female	27	47
Diagnosis at enrolment		
Ewing sarcoma	11	19
WHO grade III/IV glioma	11	19
Soft tissue sarcoma	7	12
Medulloblastoma	6	11
Ependymoma	5	9
Neuroblastoma	4	7
Osteosarcoma	4	7
Acute lymphoblastic leukaemia	1	2
Atypical teratoid rhabdoid tumour	1	2
Other ^a	7	12
Disease status at enrolment		
Primary disease diagnosis	7	12
Progressive primary disease	8	14
Relapse	41	72
Secondary malignancy	1	2
Previous therapy regimen		
Polychemotherapy ± radiotherapy	33	58
Polychemotherapy ± radiotherapy + targeted treatment	7	12
No previous treatment	7	12
Unknown	10	18
Site of origin of tissue sample		
Local to primary tumour	27	47
Metastasis	24	42
Biopsy from different sites ^b	2	4
Unknown	4	7
Time between tissue sampling and start of analysis (days) ^c		
Median	15	
Range	0–112	
Additional samples received of analysed patients		
Primary tumour sample/previous relapse		
Fresh-frozen tissue	3	5
Paraffin-embedded tissue	14	25
Later relapse/progression		
Fresh-frozen tissue	2	4

^a Other entities: adrenal hypernephroma, anaplastic multiple myeloma, germ cell tumour, myofibroblastic sarcoma, pancreatic neuroendocrine tumour, synovial sarcoma, undifferentiated sarcoma.

^b INF_14: high-grade glioma: hotspot and periphery; INF_33: neuroblastoma: primary tumour site and lymph node metastasis.

^c Only for patients enrolled from 04/2014 onwards (INF_25–INF_64).

2.3. Comprehensive molecular profiling

Paired-end libraries from tumour and germline DNA were prepared using the Agilent SureSelectXT Human V5 kit for whole-exome sequencing (WES) and, with exclusion of the enrichment step, low-coverage whole-genome sequencing (lcWGS). These were sequenced together with a tumour complementary DNA library (from poly(A)+ RNA, using the Illumina TruSeq RNA Kit v2) on an Illumina HiSeq2500 (paired-end 100 bp, rapid mode). Sequencing reads were mapped to the 1000 Genomes phase 2 human reference assembly (NCBI build 37.1) using BWA (version 0.6.2). Custom pipelines developed for use within the ICGC, as described in a recent benchmarking analysis [20], were used for detection of single-nucleotide variants (SNVs) and small insertions/deletions (InDels). Copy number variants (CNVs) were identified from lcWGS data by manual inspection. Gene fusions were predicted from RNA sequencing using deFuse [21]. Detailed bioinformatics methods can be found in the Supplementary data.

In addition, high-quality RNAs were analysed on the Affymetrix GeneChip U133 Plus 2.0 array, allowing for cross-comparison with a much larger cohort of reference tumour data than RNAseq. The expression data were visualised and analysed using the online ‘R2’ platform (<http://r2.amc.nl>). For genome-wide assessment of DNA methylation, the samples were analysed using the Illumina HumanMethylation450 BeadChip according to manufacturer’s instructions. These data were used for molecular classification by comparison with an in-house reference set.

The average costs per patient for the molecular analysis were in the range of €7000, including material shipment, data processing and storage, labor and general costs. Neither patients nor their families incurred any direct costs.

3. Results

3.1. Patient characteristics

Over a 15-month period, we received requests for 64 patients from 20 centers across Germany to participate in the INFORM pilot. Seven patients did not fulfil the inclusion criteria. Table 1 summarises the 57 enrolled patients. The average age was 13 years, with 14 young adults (>18 years) included. Sarcomas (n = 25) and brain tumours (n = 23) accounted for the majority of cases.

Most enrolled patients were in a progressive or relapse situation, but eight patients at primary diagnosis for whom no standard therapy was available were also included (non-resectable high-grade gliomas, n = 4; non-resectable or metastatic sarcomas, n = 3 and one patient with multiple myeloma).

3.2. Integrative analysis is feasible in a clinically meaningful time frame

After the first 6 months of accrual (at which point final inclusion criteria were defined), the median time from biopsy to material arrival in the processing laboratory (day 0) was 15 d (Table 1). Timings for subsequent analyses are given in Fig. 1. Tissue was suitable for analysis (sufficient amount and tumour cell content) in 52 of 57 cases (91%). DNA sequencing was performed for all 52 tumours, and RNA sequencing for 48 of 52. Thus, obtained tissue was sufficient for full profiling in most cases, including from stereotactic biopsy (e.g. diffuse intrinsic pontine glioma). Mean on-target WES coverage was 165-fold for tumour and 117-fold for control, with WGS coverage of 3.4-fold and 2.5-fold for tumour and control, respectively. RNA sequencing depth averaged 220×10^6 reads. In addition, we generated DNA methylation profiles of 49 tumours (86%) and gene expression array profiles for 46 cases (81%) (Supplementary Table 3).

Identified molecular alterations were assessed by a multidisciplinary panel with regards to biological relevance and potential druggability (i.e. whether a drug could be mechanistically linked to the alteration in a way which provided a rationale for its use). In parallel,

we created an internal database of genes for which, to our knowledge, directly or indirectly targeting drugs are in clinical development. This list incorporates information from drug development pipelines, clinical trials, and signaling pathways (see Supplementary Table 4).

Overall, the average time from tissue arrival to discussion of results in a molecular tumour board and delivery of the preliminary report to the treating center was 28 d (range 17–43 d) (Fig. 1). Except for one SNV with an allele frequency below the sensitivity of Sanger sequencing, all potentially druggable genetic alterations (i.e. 79 of 80) were verified via orthogonal methods.

3.3. Potentially druggable alterations are common in high-risk paediatric tumours

Every reported alteration was prioritised based on a 7-step scale ranging from ‘very high’ to ‘very low,’ depending on the type of alteration and its entity-specific relevance (Supplementary Table 5). We also reported biologically relevant but not druggable mutations (e.g. histone mutations in high-grade glioma) with the score ‘not applicable.’ The INFORM prioritisation system has recently been harmonised with other paediatric precision oncology programs across Europe. An overview is given in Fig. 2.

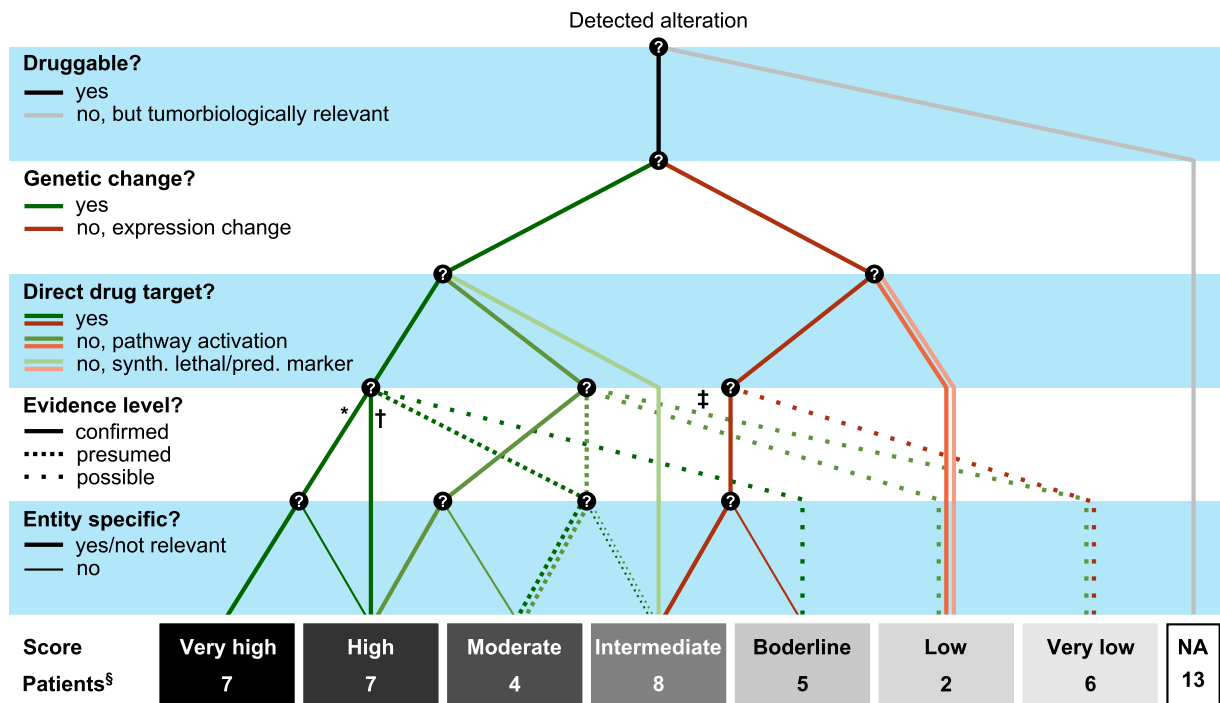


Fig. 2. INFORM target prioritisation algorithm. Druggable and tumour biologically relevant findings were prioritised and reported according to the given scheme. The remaining alterations were not reported back to the treating center. INFORM, Individualized Therapy for Relapsed Malignancies in Childhood; NA, not applicable; pred., predictive; SNV, single-nucleotide variants; synth., synthetic. ‘*’, Included molecular alterations: SNVs, small insertions and deletions (InDel), genomic translocations (fusion genes). ‘†’, Included molecular alterations: focal, high-amplitude copy number variants. ‘‡’, Genetic alterations with some modest literature evidence of possible pathway activation. ‘§’, Number of patients for which this was the highest score in their identified alterations (number for NA includes those patients where no target was identified).

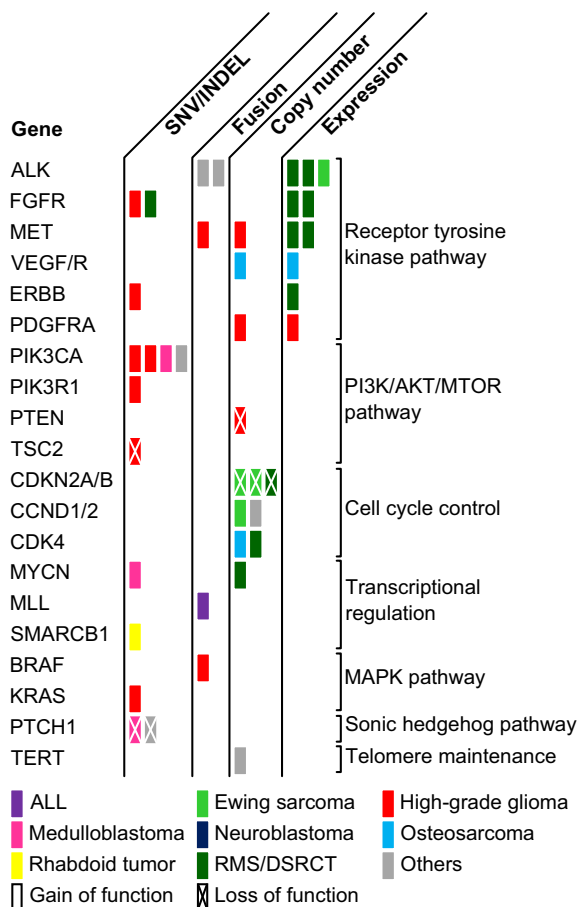


Fig. 3. All druggable alterations with a priority score of intermediate or more detected within the INFORM pilot cohort (n = 52) by whole-exome, low-coverage whole-genome, RNA-sequencing and gene expression array. ALL, acute lymphoblastic leukaemia; DSRCT, desmoplastic small round cell tumour; InDel, small insertion or deletion; RMS, rhabdomyosarcoma; SNV, single-nucleotide variants.

In total, we identified 80 potentially druggable alterations in 39 of 52 tumours (75%). Twenty-six patients (50%) had one or more alterations with priority score ‘intermediate’ or higher (Supplementary Fig. 1). Many well-known oncogenic pathways were affected, including receptor tyrosine kinases, the mitogen-activated protein kinase pathway, and the phosphoinositide 3-kinase–AKT–mammalian target of rapamycin pathway, as well as cell cycle control and transcriptional regulation (Fig. 3). Notably, most gene/pathway alterations were distributed across entities and not restricted to individual tumour types.

3.4. Analysis of spatio-temporal tumour evolution

We analysed fresh-frozen tissue from the initial diagnosis, or a subsequent relapse, from five patients (9%). The rate of retained mutations in the later sample varied widely (0–58%; Supplementary Table 6). For all pairs,

the number of non-synonymous SNVs in the relapse was equal to or higher than in the initial sample (range 1x–9.75x). In INF_37 (ependymoma), we found no overlap in mutations between the two samples (Fig. 4A), and several focal chromosomal alterations were observed in the relapse but not in the treatment-naive sample (data not shown). We additionally performed a methylation array and targeted sequencing analysis on formalin-fixed paraffin-embedded samples from primary tumours or previous/further relapses from 14 patients (25%). Methylation profiles remained stable over time (Supplementary Fig. 2), but multiple differences were observed in the CNV and SNV profiles over time (Supplementary Table 7). In certain cases, these differences involved potentially druggable alterations. The primary tumour of INF_28, for example, harbored a focal amplification of *CDK4* (a target for CDK inhibition) that was lost in the relapse (Fig. 4B). In INF_51, the primary and relapse profiles were completely different, and the assumed relapse turned out to be a secondary malignancy (glioblastoma) rather than a relapse (also confirmed histologically). A *PTPRZ1:MET* fusion in the glioblastoma, which was not present in the primary medulloblastoma, represented an interesting candidate for targeted therapy.

In two cases, different regions of a single tumour were biopsied and analysed. For both pairs, we found a remarkable difference in the number of SNVs and a mutation overlap of only 28% (INF_13) or 57% (INF_33) when comparing between samples (Supplementary Table 8).

3.5. Handling of tumour-relevant germline findings

Germline DNA of each patient was screened for damaging alterations in a predefined list of known cancer predisposition genes (Supplementary Table 2). Of 52 sequenced patients, we identified two with underlying germline mutations (4%). For both patients, this information was previously known or clinically suspected, and they had already received human genetics counselling.

3.6. Consequences of personalised tumour profiling on clinical decision making

The principle aim of this study was to develop pipelines and demonstrate feasibility, rather than systematic clinical follow-up. Some examples of impact on treatment decisions were recorded, however. In 10 of 26 patients with an alteration scored as intermediate or higher, the treating physician decided to apply a matched targeted therapy (regularly administered for more than 4 weeks), either as monotherapy or as a combination with other targeted therapies or conventional treatments. One patient was included in a clinical trial, with nine treated off-label. Furthermore, five

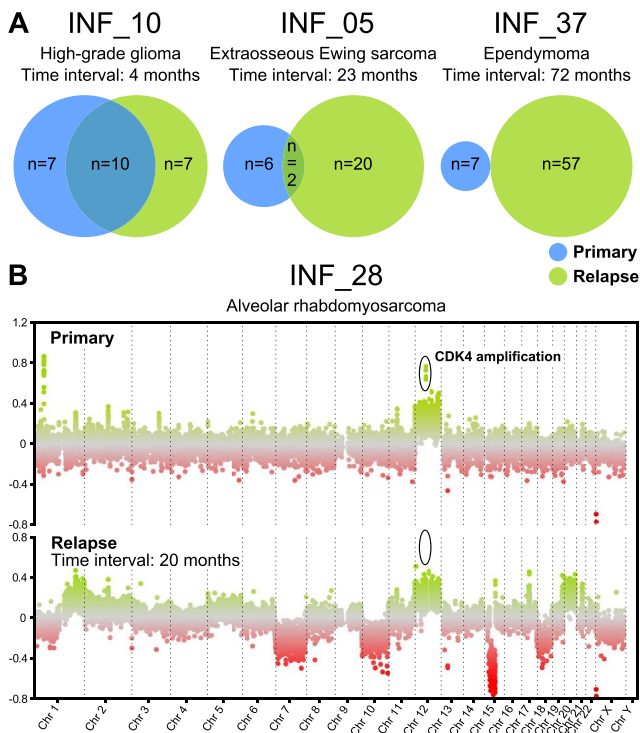


Fig. 4. Comparison of primary-relapse pairs. (A) Overlap of single-nucleotide variants between the primary and the relapse sample of 3 representative, Individualized Therapy for Relapsed Malignancies in Childhood (INFORM) pilot patients. (B) Copy number plot, generated from the 450K methylation array data, for the primary and relapse tumour of INFORM patient INF_28, demonstrating a loss of *CDK4* amplification in the relapse.

patients in our cohort showed a discrepancy between the original histological diagnosis and our molecular classification. [Supplementary Table 9](#) gives an overview of the molecular data and available clinical data of all patients.

As one example, a 12-year-old boy (INF_03) was diagnosed with a non-resectable myofibroblastic sarcoma infiltrating the skull base. Our analysis revealed a *CARS:ALK* fusion (Fig. 5A) and elevated *ALK* transcription. Although *ALK* translocations are known in myofibroblastic tumours [22], this particular fusion had not been detected by standard diagnostic tests. Based on our finding, the patient was enrolled into a clinical trial of the *ALK* inhibitor LDK378 (NCT01742286). The tumour shrank considerably after 6 weeks of treatment (Fig. 5B), allowing for a subsequent complete resection. The tumour relapsed locally and with pulmonary metastases 12 months later but responded again to the *ALK* inhibitor. Treatment is currently ongoing, 26 months after the initial analysis.

As a second example, analysis of a radiotherapy/temozolomide-resistant, progressive anaplastic pilocytic astrocytoma (World Health Organization [WHO] grade III) in a 6-year-old girl (INF_59) revealed a *BRAF* fusion, as typically found in WHO grade I pilocytic astrocytoma [23,24]. Treatment with the MEK-inhibitor trametinib in addition to empiric backbone therapy was subsequently initiated. The patient is currently in a stable disease situation 9 months after the start of trametinib treatment, with a revised diagnosis of low-grade

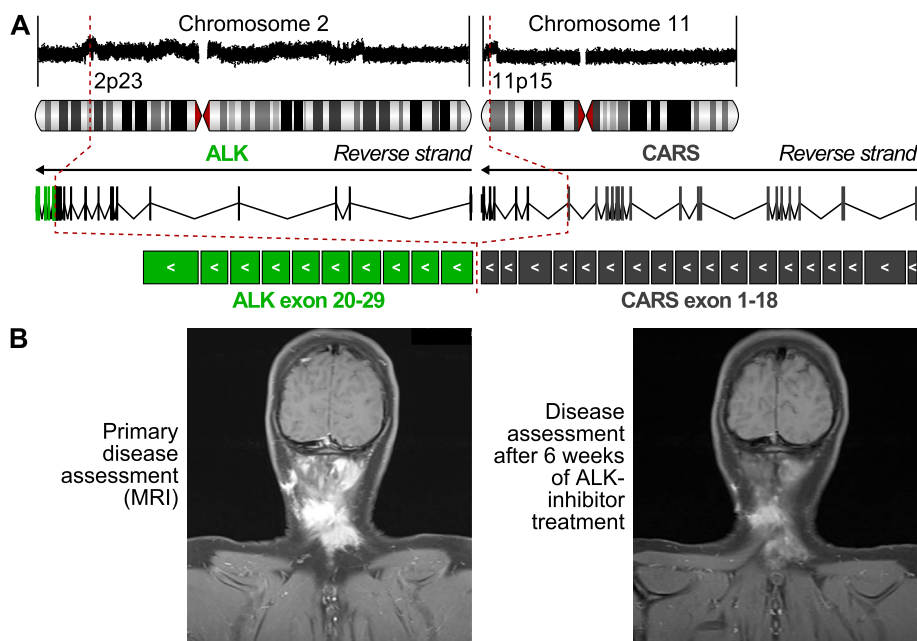


Fig. 5. Pilot patient INF_03 with myofibroblastic sarcoma. (A) Schematic image of the *CARS:ALK* fusion. The copy number plots of the involved chromosomes, generated by low-coverage whole-genome sequencing, indicate the genomic break points. (B) Magnetic resonance imaging (MRI, T1 with contrast agent, coronal view) of the tumour at diagnosis (left) and after 6 weeks of *ALK*-inhibitor treatment.

tumour with enhanced proliferative activity (supported by methylation array analysis).

4. Discussion

During this pilot phase, we established and streamlined the logistics and analytical pipelines required to overcome the various challenges of real-time clinical sequencing. We, thus, demonstrated that comprehensive molecular analysis of samples from high-risk paediatric cancer patients is feasible on a national scale and can be performed within 4 weeks.

Notably, our analyses were conducted on frozen material from a biopsy of the relapsed tumour rather than archived material. Our results support previous reports showing substantial spatiotemporal differences in the molecular profiles of multiple samples acquired from the same patient [25–27]. Relevant predictors of increased variation seemed to be a distant recurrence and a longer time interval between primary and relapse disease (possibly associated with higher exposure to different treatments in the intervening period). This is a crucial consideration in the context of clinical translation and underlines the need for tissue analysis of the current malignancy in order to adequately characterise and treat the latest disease manifestation. One caveat is that even a recent sample may not be representative for all areas of the tumour, especially with metastases. The interrogation of circulating tumour cells or cell-free nucleic acids might in future be a promising approach to pick up more subclonal events and to establish a genetic follow-up to monitor tumour evolution under therapy.

Another important feature of this study is the variety of applied methods, allowing for integrative assessment of various alterations. Besides clear driver events, we also considered variants with lower evidence (and a lower prioritisation score), such as alterations of less-studied cancer pathway members or oncogene overexpression. This is, to our knowledge, the most comprehensive approach compared with similar ongoing projects [28–33]. The current costs for the INFORM analysis are, therefore, higher than, for example, the recently published Ped-MiOncoSeq study (~\$4000) [28], although they are likely to fall to around this figure in the near future. In any case, this represents only a fraction of the complete costs of paediatric cancer care.

Our analysis also provides the opportunity to assess inter-individual variability in drug response on a genetic level. For several compounds, interactions with the so-called ‘pharmacogenes’ involved in absorption, distribution, metabolism and excretion (ADME) have been reported, and efforts are ongoing to implement this knowledge into clinical practice [34,35]. We are, therefore, establishing a systematic pharmacogenomic analysis to investigate and predict individual drug response and toxicity profiles.

The era of high-throughput genomic sequencing is triggering intense discussions on ethical aspects, such as germline sequencing and handling of incidental findings. The complexity of such issues means that there is currently a lack of consistent European guidelines [36]. In INFORM, we exclusively report (in case of consent) germline mutations that are known or very likely to elevate susceptibility to cancer (Supplementary Table 2). In this selected cohort, we detected underlying cancer predisposition in only two patients (4%), which is slightly lower than previously reported [16].

The outlook for paediatric precision oncology holds significant challenges, but also substantial potential. It is clear that the current approach of off-label or compassionate use needs to be superseded. Difficulties with accessing targeted drugs and a hesitance to use them given limited prior knowledge are hurdles to expanding a personalised treatment approach. Further challenges include small cohorts and a lack of suitable controls, unavailability of paediatric formulations, unclear toxicities, and a lack of reliable biomarkers. For some patients, the INFORM pilot results clearly influenced further treatment strategies, diagnosis and/or outcome, although for most the impact on clinical course was not substantial (gathering of detailed follow-up data was also not the main aim of this pilot phase). Thus, whilst isolated individual approaches will likely not provide significant advances [37], the generation of data on druggable alterations in relapsed paediatric tumours will provide a basis for designing interventional studies to address the need for biomarker-driven, early-phase trials for high-risk paediatric cancer patients. The fact that particular alterations were typically not restricted to individual entities supports the rationale of a cross-entity approach for these prospective studies. Although not all tumours with a specific alteration will respond identically [38], this is still a more rational way to identify biomarkers of efficacy than the traditional ‘one-size-fits-all’. To further expand our knowledge on paediatric relapsed tumour biology, and increase the patient recruitment pool, the currently ongoing INFORM registry (the follow-up to the pilot phase; <https://www.dkfz.de/en/inform/>) will soon be open to several other countries. Trials with targeted drugs are also being designed as a second phase (INFORM2 trials). These will be focused on combination strategies since pathway redundancy, tumour evolution and molecular crosstalk make it unlikely that monotherapies will result in durable responses in advanced cancers [15]. Compound selection will be complementary to other ongoing European projects (such as the ITCC AcSé-eSMART initiative) in order to provide the broadest possible opportunities for paediatric cancer patients. At a later stage, combination of targeted small molecules with, e.g. immunotherapeutics or epigenetic modifiers will pose an extra challenge but also add additional potential.

5. Conclusion

The INFORM project is a comprehensive nationwide approach to rapidly profile highly aggressive tumours of children and young adults. Sample preparation, data generation and interpretation have been optimised to allow for complete analysis within 4 weeks, enabling rapid translation into clinical decision making. In addition, the data generated give new insights into tumour evolution and possible resistance mechanisms in relapsed disease. As a next step, access to such programs must be expanded, and biomarker-driven, cross-entity phase I/II combination trials for paediatric patients are required.

Conflict of interest statement

It is the opinion of the corresponding authors that there are no conflicts of interest relevant for the subject matter of the current study. A full list of all relationships is provided in the online version of the article.

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