REVIEW

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Overview of modern genomic tools for diagnosis and precision therapy of childhood solid cancers

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Purpose of review
The application of technology and computational analyses to generate new data types from pediatric solid cancers is transforming diagnostic accuracy. This review provides an overview of such new capabilities in the pursuit of improved treatment for essentially rare and underserved diseases that are the highest cause of mortality in children over one year of age. Sophisticated ways of identifying therapeutic vulnerabilities for highly personalized treatment are presented alongside cutting-edge disease response monitoring by liquid biopsy.

Recent findings
Precision molecular profiling data are now being combined with conventional pathology-based evaluation of pediatric cancer tissues. The resulting diagnostic information can be used to guide therapeutic decision-making, including the use of small molecule inhibitors and of immunotherapies. Integrating somatic and germline variant profiles constitutes a critical component of this emerging paradigm, as does tissue-of-origin derivation from methylation profiling, and rapid screening of potential therapies. These new approaches are poised for use in disease response and therapy resistance monitoring.

Summary
The integration of clinical molecular profiling data with pathology can provide a highly precise diagnosis, identify therapeutic vulnerabilities, and monitor patient responses, providing next steps toward precision oncology for improved outcomes, including reducing lifelong treatment-related sequelae.

Keywords
functional genomics, liquid biopsy, molecular profiling, precision oncology

INTRODUCTION

Large-scale characterization of pediatric solid cancers occurred following the decoding of the human genome sequence [1] and utilized emergent next-generation sequencing (NGS) instrumentation and corresponding computational analytics. These efforts transformed our understanding of these diseases at the molecular level. While revealing that pediatric cancer genomes have relatively few somatic mutations, a dizzying array of driver alterations was uncovered including those that impact epigenetic and transcriptional programs, lead to copy number alterations, create gene fusion drivers, and confer germline susceptibility. This genomic complexity predicted that pathology-based evaluation of pediatric cancer tissues would require additional molecular assays to fully evaluate the tumor landscape and uncover variation informing disease risk, potential therapeutic response, and outcomes. As our ability to devise and utilize new methods to characterize disease complexity in the research setting has evolved, so has the understanding of how these new data types may contribute to increasingly precise diagnoses and correspondingly to personalized treatment planning. Studies demonstrating such contributions from several different platforms and analytics in the clinical trial setting have been published over the past 18 months, and this review will detail how several assay types are now being incorporated into clinical diagnosis and treatment

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KEY POINTS

- Prospective studies of pediatric solid cancers substantiate the use of multiple assays that profile DNA and RNA to produce a comprehensive data set to inform diagnosis.
- Next generation sequencing (NGS)-based profiling of DNA from constitutional, nondisease involved tissue can identify germline susceptibility.
- NGS-based profiling of DNA from disease-involved tissue may identify therapeutic vulnerabilities, copy number alterations that inform prognosis, or tumor mutational burden indicative of response to immune checkpoint blockade therapy.
- RNAseq from disease-involved tissue may be analyzed by numerous approaches to identify driver fusions, outlier gene expression and alternative splicing events.
- DNA from CNS tumor tissue can be assayed to produce genome-wide methylation data that, when evaluated by a machine learning-based classifier, provides accurate classification to augment conventional pathology.

planning for children with solid tissue malignancies.

OVERVIEW OF GENOMIC TOOLS

Methylation profiling of tumor DNA. Methylation patterns of the human genome in different tissues are unique and may become altered in disease-specific ways within cancer cell genomes. These facts have led to large-scale efforts to catalog the genome-wide methylation patterns of different tumor types from known tissues of origin based on diagnoses from conventional pathology, using arrays of CpG loci genome-wide. The resulting data were used to develop machine learning-based diagnostic classification schema. These schemas then can be used to evaluate methylation data from any newly assayed tumor DNA, yielding a diagnostic classification and assigning a confidence score for the resulting diagnosis that conveys the certainty of the derived classification. Further value from methylation array data analysis includes the evaluation of copy number alterations genome-wide based on the CpG loci represented on the array and their corresponding chromosomal locations (Fig. 1a). Similarly, in central nervous system (CNS) malignancies, evaluating Methylguanine-DNA Methyltransferase (*MGMT*) methylation status from the array is critical to decision-making for the use of temozolomide chemotherapy in high-grade disease. Finally, clustering approaches such as principal components analysis (PCA), t-distributed stochastic neighbor embedding (tSNE), or Uniform Manifold Approximation and Projection (UMAP) can be used to provide a visual cluster-based evaluation of an individual patient sample relative to other prior diagnoses (Fig. 1b).

Retrospective analyses of large CNS cancer cohorts have demonstrated the robustness of methylation-based classifier approaches [2,3] and their ability to provide more precise diagnostic information in the setting of indeterminant diagnoses, to the extent that the 2021 WHO Guidelines for diagnosis of CNS malignancies included methylation-based classification within the standard of diagnosis [4]. Recently, a large prospective trial of methylation array-based classification was reported for pediatric patients with CNS malignancies, further demonstrating improved precision in sub-group classification (e.g., higher granularity among similarly subtyped classes of CNS cancers) and linking new sub-group classifications to outcomes [5▪▪].

Similar approaches have been demonstrated to classify sarcomas of different types using retrospective cohorts [6], as also was reported for neuroblastomas [7]. Although these classification schemas have not yet been included in WHO-guided diagnostic criteria, this is likely in the near future.

Tumor RNA characterization. RNA isolated from solid tumors and sequenced using NGS methods provides a rich source of information that can be evaluated by multiple analytic pipelines to yield valuable information. Clinically, this has been limited to the identification of gene fusions, to verifying predicted impacts of splice-site mutations on alternatively spliced transcripts, and to correlating over-expression with amplified copy number, or absence of expression due to deleted genes or nonsense-mediated decay. Databasing of over 12 000 RNAseq expression profiles from pediatric cancers has greatly aided diagnosis by virtue of online tools and data display such as that hosted at the Treehouse Childhood Cancer Initiative (https://treehousegenomics.ucsc.edu). An example of this type of comparison is shown in Fig. 2, wherein a single sample of indeterminate diagnosis is localized by virtue of its RNA expression profile in proximity to clustered profiles of samples with known diagnosis, through PCA.

Recently, Shlein *et al.* [8▪▪] reported an intriguing method based on RNAseq data to molecularly define most childhood cancers and accurately predict subgroups and corresponding outcomes. Their methods measured transcriptional entropy and demonstrated significant diversity both between and within tumor types, in contrast to the relatively quiet genomic DNA landscape of most pediatric cancers. They then leveraged this transcriptional

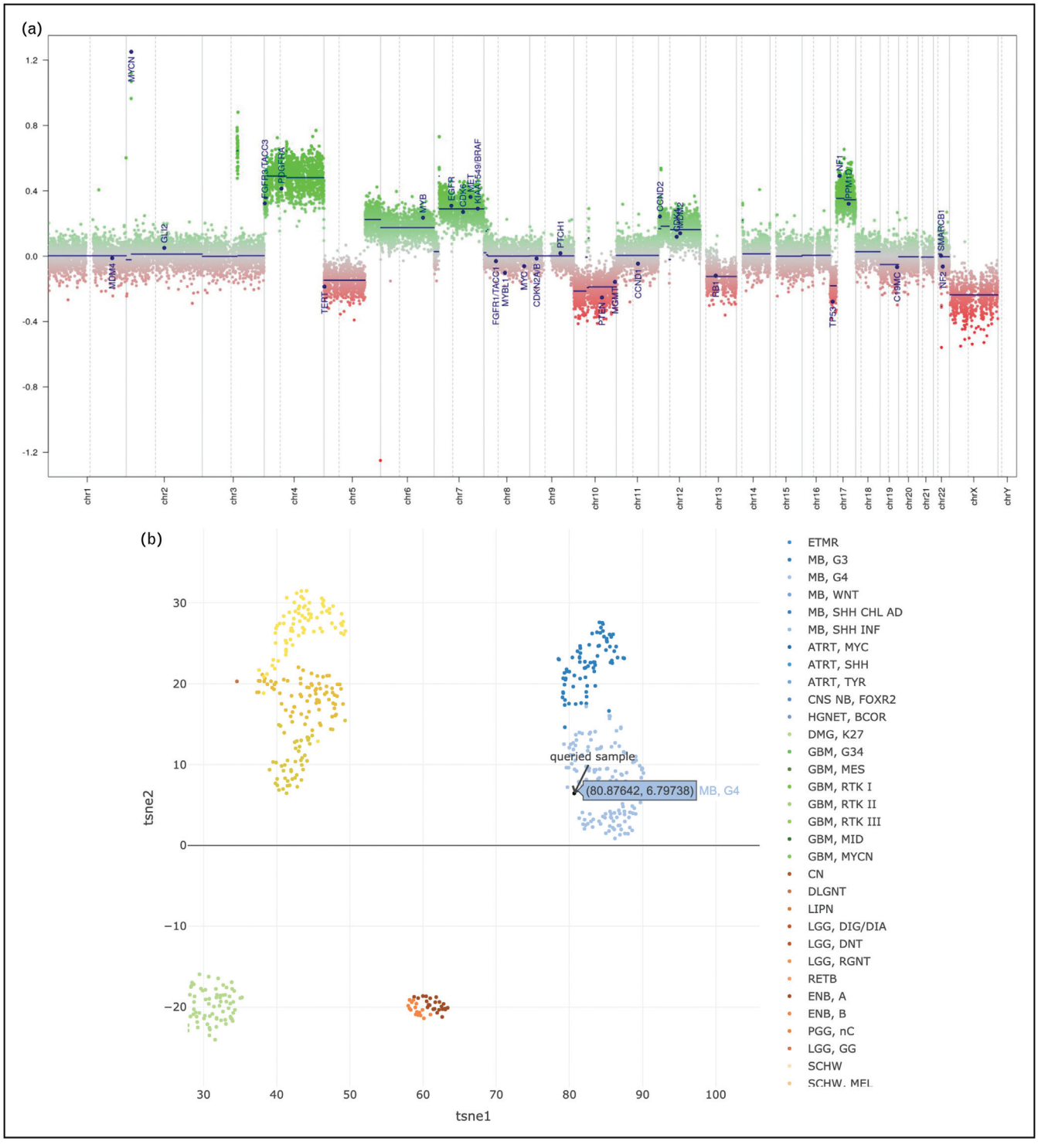

FIGURE 1. Output analyses from genome-wide methylation array data. Figure a illustrates a genome-wide copy number variant plot from a patient diagnosed with a Group 4 medulloblastoma. Figure b illustrates the methylation array data from this patient on a tSNE projection, grouped with data from other Group 4 medulloblastoma diagnoses.

variability to improve diagnosis based on a clustering approach that performed unsupervised classification of RNAseq data to produce an atlas of 455 tumor and normal tissue classes based on gene expression similarity. An ensemble of convolutional neural networks was designed to provide the ability to robustly classify data from newly studied tumors, which was refined using their classifier in 7% of the cases examined. Further clinical validation of such an approach could represent a unique diagnostic

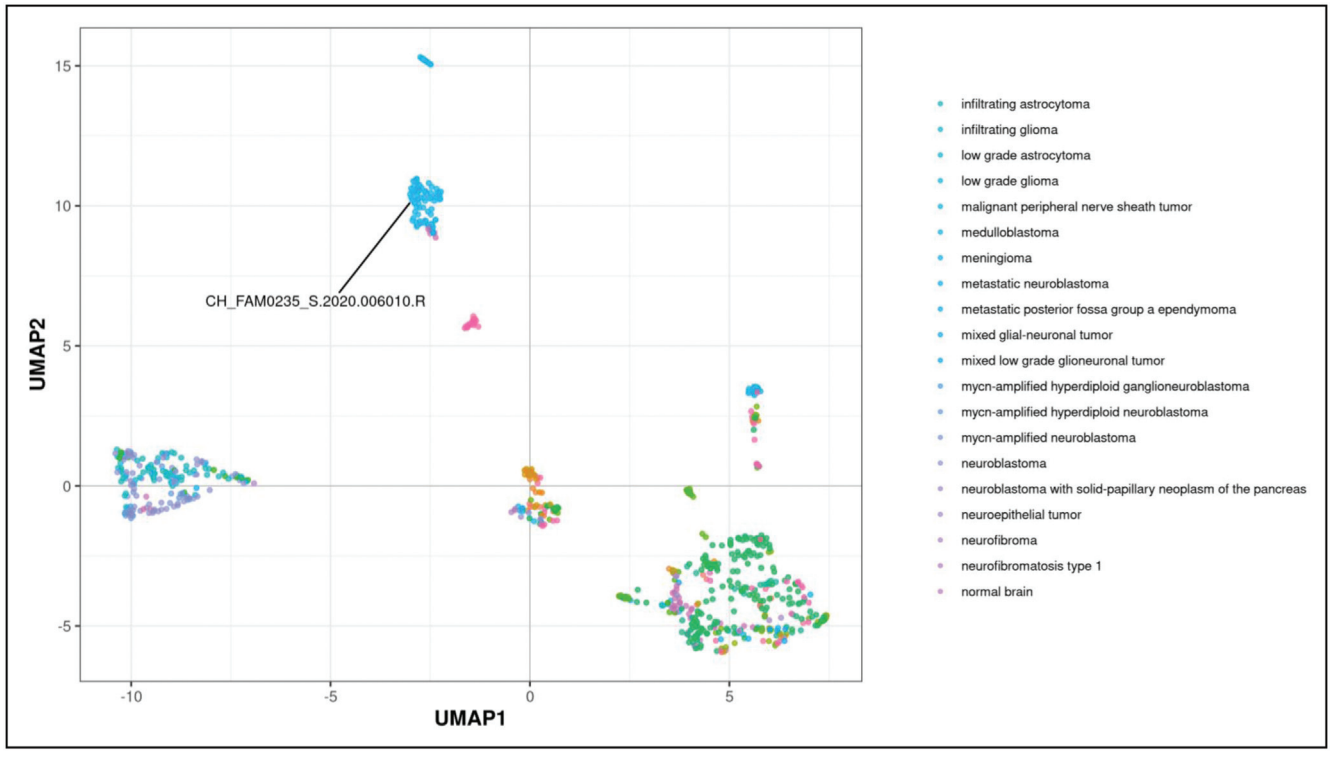

FIGURE 2. RNA expression comparison to known diagnoses. Figure illustrates co-clustering based on RNA expression profiling from RNAseq, for a patient diagnosed with Group 4 medulloblastoma.

approach to classification that could be applied more broadly to identify gene fusions, alternatively spliced transcripts and outlier expression from a single dataset.

NGS-based identification of somatic and germline alterations. NGS has dramatically improved the detection of many types of variants present in germline and somatic DNA and, with decreasing costs of data generation and increasing sophistication of computational variant identification, has become more broadly utilized in molecular profiling of pediatric cancers. Moreover, the downstream impact of identified variants on diagnosis and clinical care, as described below, has established the significant value of these assays. Recent reports include the iTHER prospective trial at the Princess Maxima Center in the Netherlands which used comprehensive molecular profiling [9▪] demonstrated feasibility and impact of this approach to inform diagnostic, prognostic and therapeutic targets, along with germline susceptibility. This trial result led to the adoption of whole exome and RNA sequencing as the standard of clinical care, including at primary diagnosis, within this tertiary care site for pediatric cancers. As cited previously, the DKFZ INFORM prospective trial of CNS malignancies that combined methylation array-based classification with a gene panel to evaluate germline and somatic mutations and RNAseq for fusion identification has driven the approval for reimbursement of this comprehensive testing regimen by German government-funded medical insurance [5▪▪]. In the United States, the GAIN consortium tested pediatric patients with extracranial solid tumors using a gene panel test of somatic DNA that was capable of detecting mutations in several hundred cancer genes, including copy number alterations and a limited number of gene fusions. In this setting, 77% of the 209 patients with a diagnostic finding had gene fusions. Hence, the study conclusion was that targeted panel testing that includes the ability to identify gene fusions had substantial clinical benefit for these patients [10▪]. A U.S.-based pediatric molecular profiling project, the Molecular Characterization Initiative (MCI), sponsored by the National Cancer Institute (NCI) opened to enrollment in 2022. In this project, cancer patients from birth to 25 years of age, diagnosed with CNS cancers, soft tissue sarcomas or a collection of rare cancer types at hospitals affiliated with the Children's Oncology Group (COG) receive comprehensive clinical molecular profiling (methylation array, fusion panel testing, tumor vs. normal exome testing) and return of results within 21 days of receipt of tumor and blood samples. Additional cancer types will be eligible for participation over the next 4 years

of this 5-year project (https://www.cancer.gov/research/areas/childhood/childhood-cancer-data-initiative/data-ecosystem/molecular-characterization). Importantly, all de-identified data and results are actively being deposited into the Childhood Cancer Data Initiative [11]

Germline susceptibility. One example of an NGS finding is the identification of germline-based cancer susceptibility, present in over 10% of all pediatric cancers, but ranging up to 15% in specific tissue site diagnoses. Knowledge of inherited or de novo cancer susceptibility has logical impacts on cancer survivorship care and reflex testing within family members, but more recently has been studied in the setting of cancer treatment with immune checkpoint blockade inhibitor (ICBI) therapies [12▪▪,13▪▪]. The results of clinical trials in pediatric and AYA patients with high or ultra-high tumor mutational burden (TMB) due to constitutional mismatch repair deficiency (CMMRD) or Lynch Syndrome-associated solid cancers being treated with anti-PD1 ICBI monotherapy or combined therapies (anti-CTLA4 plus anti-PD1 or MEK inhibition plus anti-PD1) have demonstrated durable responses in >50% of patients within an admittedly rare subpopulation of pediatric cancers typically having dire outcomes. Importantly, high TMB (>5 mutations/Mb) and/or high microsatellite insertion (MSI) index are strong predictors of response, as are blood-measurable immune parameters such as the level of 4–1BB positive CD8 T cells and elevated TCR clonal diversity [13▪▪].

Somatic indicators of therapy response. Due to the types of variants identifiable from NGS-based testing, and the increasing numbers of FDA- and/or EMA-approved targeted therapies, genomic profiling information can inform cancer treatment decision-making. One such impact is the identification of tumor-specific alterations to known cancer driver genes for which there are existing targeted therapies or agents under investigation in clinical trials. However, despite large-scale characterization of pediatric cancer genomes, there are typically variants identified in cancer-related genes for which no known functional impact on cancer onset or progression can be discerned. This reality applies even to the most frequently mutated genes in cancer such as TP53, although exciting new approaches to contextualizing variants in this gene have been recently published [14,15].

1. *Protein-targeted therapies*. One paradigmatic shift brought about by genomic profiling of adult cancers has been the emergence of small molecule and antibody-based therapies that are highly specific for driver genes. This shift has become manifest in the clinical offering of gene panel testing using DNA derived from cancer samples (needle biopsy or resection) that can identify whether known pathogenic driver mutations in genes with one or more FDA approved targeted therapies are present. Importantly, the discovery of new driver genes and alterations from adult cancer genomics has resulted in a plethora of new therapies that directly target the resulting altered proteins with reduced adverse effect profiles and result in emerging standard-of-care treatments through successful clinical trials. Unfortunately, the overlap in drivers between adult and pediatric cancers is small [16] and hence the benefit of genomic testing to predict targeted therapy response in pediatric cancers suffers from this deficit. Furthermore, since driver alterations are of variable types across pediatric diagnoses, a simple gene panel test may not be capable of detecting all types of alterations, especially copy number variants or fusion genes.
2. *Prognostic markers of risk*. Recognizing the lifelong impact of aggressive chemo- and radiotherapy treatment in the pediatric setting, efforts to investigate dose reduction in lower risk outcome subtypes has been pursued through clinical trials over the past decade. In trials where these risk predictors were identified as relevant to determining dose reduction and improved long-term sequelae and quality of life, they have been implemented into the diagnostic rubric to determine care. Hence it is important that NGS-based DNA profiling assays produce clinically relevant information about prognostic specific amplification and deletion status genome-wide.
   Multiassay testing regimens have been slow to develop yet, in settings where these have been pursued, there is clear clinical benefit to pediatric patients from the aspects of (i) identifying targeted therapy options for known driver alterations, (ii) detection of germline susceptibility, which can be interpreted clinically in multiple ways, to (iii) identification of focal, arm-level or whole chromosome copy number alterations, which may provide established prognostic information from clinical trial-based results, and indicate the modification of treatment aggressiveness accordingly [6,17–20].
3. *Immune checkpoint blockade inhibitor therapies*. The development of immune therapies that inhibit various immune checkpoints has transformed cancer care in the adult setting for high mutation-load tumor types such as smoking-associated lung cancers, POLE-mutated endometrial cancers, and MSI-instable colorectal cancers, among others. Adult clinical trials of these agents have been the first to achieve FDA approval in a

tissue-agnostic setting, by enrollment and efficacy demonstration in the setting of high or ultra-high TMB regardless of tissue of origin [21]. In the pediatric setting, however, high and ultra-high mutation loads are certainly the exception but do occur in those with constitutional mismatch repair defects (CMMRD), Lynch syndrome and Li-Fraumeni syndrome. Recently reported clinical trials of these agents, as cited above, have resulted in durable complete and partial responses in these patients regardless of tissue site.

"Functional genomics": therapy response evaluations. Despite the broader implementation of multiomic methods in characterizing pediatric cancers, identifying appropriate treatment is not always clear. For example, many fusion drivers including transcription factor fusions lack any targeted therapies, whereas somatically "quiet" DNA profiles may offer no clues as to likely response to any type of therapy. In this regard, the use of rapid functional testing of therapeutic response obtained by exposing tumor cells to a panel of chemotherapies and small molecule inhibitors is emerging as a clinical approach that indicates likely response. One such approach using an ex-vivo drug sensitivity profiling assay (DSP) was described recently [22▪▪]. Here, spheroid cultures from fresh tumor tissues were grown over a 3-week period in 3D culture conditions in 384 well plates prespotted with 75–78 chemotherapies and small molecule inhibitors. Patients on this study were simultaneously profiled by gene panel testing of tumor and normal DNA, by tumor RNAseq and phospho-proteomic mass spectrometry as well as methylation array classification. Study results demonstrated that ex-vivo DSP produced the same drug targets as molecular profiling. Importantly, drug vulnerabilities were identified in 80% of cases lacking actionable (very) high-evidence molecular events, adding value to the molecular data.

CONCLUSION

Multiple prospective studies now support the hypothesis that combined clinical testing of DNA and RNA from pediatric solid cancers refines diagnosis, identifies therapeutic vulnerabilities, and uncovers germline susceptibility when present. These data combine with conventional pathology-based evidence to render precision diagnosis and, when the data are shared, may benefit other patients and support future discoveries. Significant barriers to progress remain, however. These include (i) inability to interpret novel variants or variants of uncertain significance and their contribution to cancer development, prognosis or therapy selection, (ii) lack of definitive treatment guidance from genomic testing results, (iii) lack of driver-specific targeted therapies for unique pediatric cancer drivers. The first barrier is currently being approached by the Atlas of Variant Effects Alliance (https://www.varianteffect.org/), a consortium that is systematically mutating sites across many genes and evaluating their impact via functional readouts, thereby creating comprehensive variant effect maps of human genes. A more focused approach might prioritize which of the most frequently mutated positions in cancer-relevant genes should be functionally characterized, using AI-based protein structure-function prediction, for example, AlphaFold2 [23] for further study. The second barrier could be addressed using real-time functional screening approaches such as the ex-vivo screening of disrupted tumor cells cited above, although this is limited to screening only available therapies. When these assays are performed in the context of broad multiomic testing, and the indicated therapy or therapies are used to treat the patient, the possibility to develop artificial intelligence-based predictive methods from large databases of treated patients with known outcomes, holds significant promise toward automating these predictions. The third barrier is challenged by the need to identify suitable medicinal chemistry approaches to design therapies, especially for transcription factor fusions and epigenetic drivers as well as undruggable targets (MYC, for example). This important effort should be encouraged by making funding available to support pursuit of novel concepts. Downstream of encouraging preliminary data, the engagement of pharmaceutical and biotechnology companies alongside pediatric-focused cooperative groups will be needed to support the clinical trials to test these novel therapies.

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None.

Conflicts of interest

There are no conflicts of interest.

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- ▪ of special interest
- ▪▪ of outstanding interest

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This prospective study combined data from more than 1200 pediatric brain tumor patients to demonstrate the feasibility of integrating DNA methylation profiling with DNA-based targeted sequencing results to facilitate clinical histopathologic diagnoses. The study also emphasized the importance of identifying fusion drivers from RNA when indicated by methylation or histopathology, and of studying the germline genome in the context of identifying cancer predisposition, which drives about 10% of pediatric brain cancer development. This study further emphasizes the dearth of novel therapies unique to pediatric cancer drivers, identifying that only 15% of alterations identified by targeted sequencing had direct implications for therapeutic response.

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This study used an optimized, multilevel approach to cluster RNAs from over 13,300 pediatric cancer transcriptomes, creating an atlas and to compare the clustered tumor groups to conventional diagnosis approaches in the context of disease outcomes. Subgroups of cancers with established conventional diagnosis emerged from this clustering approach that better predicted disease outcomes, potentially enabling reproducible diagnostic information. By observing the greater transcriptional diversity in pediatric compared to adult cancers, an ensembled convolutional neural network classifier was devised. This classifier was applied to a prospective cohort of pediatric cancers and was able to match or clarify diagnosis for 85% of the samples evaluated. The results of this study set up the potential for an RNA-based classifier that could simplify clinical molecular diagnosis with data from a single assay (RNAseq), which could further inform gene fusion identification, outlier expression, pathway analysis, and other results.

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This paper describes a prospective precision oncology program in children and adolescents, conducted in the Netherlands between April 2017 and 2021, employing low coverage whole genome sequencing, exome sequencing, RNA sequencing, Affymetrix and/or 850K Illumina methylation profiling of 226 patients. The study indicated germline pathogenic variants in 16% of patients, had a high yield (90.3%) of druggable somatic alterations and led to either revised or refined diagnosis in 3.5% of patients. Importantly, the trial results led to the adoption of molecular profiling as standard clinical care at the Princess Maxima Center for all childhood cancers, including at primary diagnosis.

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This prospective observational cohort study evaluated the clinical impact of molecular tumor profiling using targeted sequencing panel testing of extracranial solid tumors from 12 U.S.-based institutions. Of 298 patients, 86% had one or more alteration with potential impact on care, including diagnostic, prognostic or therapeutic information. Response to targeted therapy receipt of a small subset of patients was reported, majority of which had druggable gene fusions.

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This international consortium registry study reports on the use of immune checkpoint inhibitor treatment of 45 progressive or recurrent tumors from 38 patients with mismatch repair or polymerase proofreading deficiencies. Most patients had durable objective responses, with a 3 year survival of 41.4%. The observation of pseudo-progression was common and associated with intratumoral and systemic immune activation, yet patients who continued treatment despite pseudo-progression achieved durable responses.

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This manuscript describes a small cohort of pediatric cancer patients that were treated with nivolumab based on high mutation burden from NGS testing assay results and showed dramatic responses in aggregate. Importantly, the patients were also monitored by blood-based assays including the evaluation of T cell receptor repertoire and flow cytometry. Correlative studies indicated that children with higher total indel and microsatellite indel burden had improved survival, and that blood markers such as higher 4-1BB+ CD8 T cells at baseline, lower regulatory T cells in blood at baseline, and a more diverse TCR-beta clonality were more likely to have durable response to nivolumab therapy.

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This paper outlines the results of a two-year study associated with the INFORM clinical trial, wherein ex vivo drug sensitivity profiling was performed using tumor spheroids from patient cancer samples and a library of ~75 clinically relevant drugs. Drug vulnerabilities were identified within three weeks, and in 80% of cases lacking actionable molecular findings from INFORM profiling assays, with similar responses in selected patients closely mirroring the drug screening results.

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