Clinical and immunophenotype correlating with response to immunotherapy in paediatric patients with primary liver carcinoma. A case series

Allison F. O'Neill,[a,*] Alanna J. Church,[b] Angela Feraco,[a] Jennifer Spidle,[a] Catherine B. Wall,[a] Heung Bae Kim,[c] Scott Elisofon,[d] Khashayar Vakili,[c] Max Pimkin,[a] Neekesh V. Dharia,[e] Nathan R. Shelman,[f] Antonio R. Perez-Atayde,[b] and Carlos Rodriguez-Galindo[g]

[a]Dana-Farber Cancer Institute/Boston Children's Cancer and Blood Disorders Center and Harvard Medical School, Department of Pediatric Oncology, Boston, MA, USA
[b]Boston Children's Hospital and Harvard Medical School, Department of Pathology, Boston, MA, USA
[c]Boston Children's Hospital and Harvard Medical School, Department of Surgery, Boston, MA, USA
[d]Boston Children's Hospital and Harvard Medical School, Department of Hepatology, Boston, MA, USA
[e]Genentech, South San Francisco, CA, USA
[f]University of Kentucky, Department of Pathology, Lexington, KY, USA
[g]St. Jude Children's Research Hospital, Departments of Global Pediatric Medicine and Oncology, Memphis, TN, USA

eBioMedicine
2024;104: 105147
Published Online xxx
https://doi.org/10.1016/j.ebiom.2024.105147

Summary

Background Paediatric hepatocellular carcinomas (HCC) traditionally arise in the context of a normal structural and functional liver and carry a dismal prognosis. While chemotherapy is the frontline standard, there is emerging interest in the study of immunotherapies for paediatric patients with relapsed/refractory disease. There is limited data to support whether immunotherapies will be of utility in this patient population.

Methods Six paediatric patients (median age:16 years, range: 12–17 at the time of treatment) with advanced hepatocellular neosplams, either conventional hepatocellular or fibrolamellar carcinoma, were treated with immunotherapy. Patients were consented to institutional genomic profiling and biobanking protocols. Baseline samples and serial tissue samples, when available, were evaluated for somatic mutation rate, actionable gene mutations, and pan-immune bulk RNA expression profiling. Results were correlated with clinical course.

Findings Three patients responded to checkpoint inhibition: one achieved a complete, durable response and the other two, prolonged stable disease. Three additional patients progressed. Diagnostic tissue from the complete responder demonstrated a higher relative mutational burden and robust immune infiltrate. Pre-treatment samples from the three responders demonstrated decreased expression of genes associated with T-cell dysfunction.

Interpretation A subset of patients with primary paediatric hepatocellular tumours will respond to immunotherapy. Immunotherapies are currently under prospective study for relapsed/refractory liver tumours in paediatric patients. Results from this report support the prospective collection of serial serum and tissue samples which may further identify genomic and immunophenotypic patterns predictive of response.

Funding This work was supported by Philanthropic funds (Pan Mass Challenge, Team Angus and Team Perspective).

Keywords: Hepatocellular carcinoma; Pediatric; Immunophenotype; Genomics

Introduction

Hepatocellular carcinoma (HCC) is the second most common primary paediatric liver malignancy diagnosed predominantly in adolescents and young adults.[1] Hepatocellular carcinomas in children are divided into two categories based on disease histology: conventional hepatocellular carcinoma (HCC) and fibrolamellar carcinoma (FLC). In contrast to hepatocellular tumours diagnosed in older adults, the majority of conventional paediatric HCC tumours arise *de novo* in the context of a structurally normal liver. Only 20% of cases arise secondary to cirrhosis and preliminary studies demonstrate

*Corresponding author. Dana-Farber Cancer Institute, 450 Brookline Avenue, Boston, MA 02215, USA.
E-mail address: allison_oneill@dfci.harvard.edu (A.F. O'Neill).

Research in context

Evidence before this study

A PubMed search was performed to source data for paediatric patients treated with checkpoint inhibition or combination immunotherapies identifying a paucity of evidence for the use of these agents across paediatric solid tumours and, in particular, for paediatric liver carcinomas. A companion search was performed to identify known predictors of immunotherapy response in adults.

Added value of this study

This report identifies paediatric patients with malignant hepatocellular tumours who responded to immunotherapy and pairs therapeutic response with disease characteristics, immune, and genomic phenotype.

Implications of all the available evidence

Results to this report support the ongoing or future study of immunotherapies in paediatric patients with liver carcinomas along with longitudinal serum and tumour assessments of immune function to further validate these findings prospectively and in a larger cohort.

paediatric HCC tumours to have a unique, non-overlying genotype with adult tumours.[2,3] FLC tumours consistently harbour a *DNAJB1::PRKACA* chimeric fusion and uniformly arise in the context of a normal liver.[4] Both entities are exceedingly rare in children and young adults and are predicted, in aggregate, to occur with a frequency of approximately 100–150 cases/year in the United States.[5] Outcomes for patients who present with upfront, resectable disease are excellent with an approximate 85% 5-year overall survival.[6] The majority of patients, however, present with advanced disease precluding resection and resulting in dismal outcomes.[6]

The treatment of primary liver carcinomas in children is dictated by histology. Conventional HCC tumours, in contrast to adult HCC tumours, respond to platinum- or doxorubicin-based chemotherapy regimens up to 50% of the time.[7] For this reason, chemotherapy is the upfront standard of care for patients with unresectable tumours often in combination with tyrosine kinase inhibition.[8] Despite chemotherapeutic response, the majority of tumours do not shrink adequately enough to facilitate definitive local control, which remains a requisite for cure. Fibrolamellar carcinomas, perhaps by virtue of a shared genomic alteration, behave similarly in both paediatric and young adult patients. Surgical resection of disease remains paramount for cure and there is no successful standard for systemic therapy.[9]

Checkpoint inhibitors have been extensively studied for adult patients with advanced HCC and are now FDA-approved as first-line treatment, in combination with bevacizumab, for unresectable liver cancer in adults.[10] Checkpoint inhibitors, in combination with vaccine (NCT04248569), or 5-FU and interferon (NCT04380545) are currently under study for patients ≥ 12 years of age with FLC. Pembrolizumab has been prospectively studied in paediatric patients with relapsed or refractory solid tumours. Geoerger et al. published a series of paediatric patients treated on the KEYNOTE-051 trial; three patients enrolled had hepatocellular carcinoma and all progressed.[11] The study was not intended to report on granular details of disease or biomarkers of response.

Checkpoint inhibitors have gained minimal traction as a therapeutic for paediatric patients given uniformly low tumour somatic mutation rates.[12] However, paediatric liver carcinomas are more genomically complex than most paediatric tumours and may be more apt to respond to immunotherapies than most paediatric tumours.[13] Given the poor prognosis associated with paediatric HCC, and the paucity of available data supporting immune therapies in children, we sought to more deeply explore the clinical course and immuno- and genomic phenotype of a series of these patients treated with immunotherapy.

Methods

Patients

Paediatric patients with a confirmed diagnosis of advanced (unresectable or metastatic) hepatocellular carcinoma (conventional or fibrolamellar) who presented to the Dana-Farber Cancer Institute between May 2015 and August 2016 were offered treatment with checkpoint inhibition—alone or subsequently in combination with other drugs—following upfront chemotherapy or observation post-resection. All patients were ≥ 12 years age at the time of treatment therefore treatment plans were designed per adult standards for medication administration, toxicity assessments, and dose adjustments. Informed consent was obtained prior to the treatment of all patients. All patients had RECIST 1.1 measurable or evaluable disease at the time of treatment. Patients were not previously treated with any form of immunotherapy, were not receiving chronic steroids or immunosuppressive medications, did not have known autoimmune disease, a history of allergic reaction to similar agents, or uncontrolled intercurrent illnesses. Immunotherapy was not administered to patients with a prior solid organ transplantation.

Treatment

Patients were uniformly started on PD-1 inhibitor monotherapy. At the time of progression, additional

agents (bevacizumab or vaccine) were added or consideration was given to dual checkpoint inhibition. At the time patients were receiving treatment, there was limited safety data and no existing efficacy data for checkpoint inhibition in paediatric patients. Each regimen was therefore tailored to the clinical context, patient/family preference, and availability of safety data in adults.

Patients received one of the following checkpoint inhibitor therapies: 1) pembrolizumab (2 mg/kg, max dose: 200 mg) infused over 30 min every three weeks or 2) nivolumab (3 mg/kg) infused over 60 min every three weeks when in concert with ipilimumab (1 mg/kg, infused over 90 min), or infused every two weeks when administered as monotherapy. After a prolonged course of pembrolizumab monotherapy, one patient received pembrolizumab in combination with a personalized synthetic peptide vaccine and later with bevacizumab (15 mg/kg), the latter administered every three weeks. Patients were permitted to undergo local control measures while receiving immunotherapy provided residual target lesions could be followed. Per specifications in the pembrolizumab drug insert, patients could receive pembrolizumab therapy for up to 2 years. When given in combination, nivolumab and ipilimumab were administed for a total of four cycles reverting to nivolumab monotherapy thereafter for up to 2 years.

Response evaluation

Individual patient response was evaluated on serial imaging studies (abdominopelvic MRI (where appropriate) with Eovist and chest CT) performed every 2–3 cycles, and, for patients with secreting tumours, serial tracking of alfa-feto protein levels (AFP). Criteria to continue treatment included stable disease (SD), partial response (PR), or complete response (CR) in the absence of toxicity. Baseline physical exams, organ function, AFP levels, and imaging studies were performed prior to initiating therapy. RECIST criteria 1.1 was utilized to document response. AFP response was analyzed in conjunction with the timing of therapy and intervening local control procedures.

Genomic evaluations

OncoPanel, a next-generation sequencing panel examining over 400 genes for single nucleotide variants and small insertions or deletions commonly implicated in paediatric cancer, was performed on diagnostic or pre-immunotherapy specimens when available. Oncopanel is also able to evaluate tumour mutational burden and mutational signatures, as previously described.[14,15] Genomic alterations were reported utilizing the Association for Molecular Pathology (AMP), the College of American Pathologists (CAP), and the American Society of Clinical Oncology (ASCO) guidelines to denote clinical relevance.[16] The Boston Children's Hospital Solid and Brain Tumour Fusion Panel, using ArcherDX technology as previously described, was run on each specimen to confirm the presence or absence of a *DNAJB1::PRKACA* fusion.[17]

Evaluation of the immune infiltrate

Diagnostic or pre-immunotherapy tissue, when available, was analyzed as was tissue from subsequent local control timepoints. Slides were stained for human CD45 (a receptor expressed on all leukocytes), CD3 (to specifically assess the proportion of leukocytes that are T-cells), and PD-1/PDL-1 expression and cell counts were tallied at 200x by one reviewer who was blinded to the treatment timepoints. Slides were characterized as having "few" CD45 positive cells if counts were <200 cells, "moderate" if 200–500, and "prominent" if > 500. A Nanostring Technologies pan-cancer immune panel was run on available tissue from each timepoint utilizing the PanCancer Immune Oncology panel of 770 genes. RNA expression was measured with the nCounter technology according to the manufacturer's protocol (NanoString Technologies, Inc. Seattle WA).

Ethics

All patients treated were simultaneously consented to an Institutional Review Board (IRB)-approved internal protocol, DFCI #17-000, which allowed for genomic profiling of tumour tissue. Under a separate IRB-approved Dana-Farber Protocol #17–086, further analysis of patient specimens was performed to interrogate the immune infiltrate by immunohistochemistry and Nanostring Technology. Consent was obtained from the participants or their legal guardians and, per institutional standards, assent was obtained for patients greater than or equal to 10 years of age.

Toxicity

Toxicities were graded as per CTCAE 5.0. Dose-adjustments and initiation of corticosteroids or other immunosuppressive drugs for immune-related adverse events (AEs) were pursued as per the JCO consensus guidelines published in 2017.[18]

Statistical methods

Clinical characteristics (age, sex, extent of disease, treatment, toxicities to therapy, response rates, life status), immunohistochemistry (staining for CD45, CD3, PD1, and PDL1), and genomic findings (panel sequencing for tumour mutational burden, DNAJB1:PRKACA fusion status, gene variants and copy number alterations) were tabulated descriptively. Age, at diagnosis and at the time of treatment with immunotherapy, was reported as the median plus the interquartile range (IQR; Q1, Q3). Given non-normally distributed data, the Wilcoxon rank sum test was performed to compare immune infiltrate counts between tissue types recognizing definitive limitations in

powered conclusions given a small sample set (p-value < 0.5 denoting significance). A comparison of the means with report of 95% confidence intervals was further performed to lend clinical relevance to these results. NanoString data was analyzed utilizing the ROSALIND© platform which allows bioinformatics reporting on quality control, normalization, pathway analysis, and differential gene expression. The cut-off for a statistically significant change in gene expression was selected a priori as a 2.5-fold increase or decrease with a p-value of <0.5.

Role of funders

The funders did not play a role in study design, data collection, data analyses, interpretation or writing of the report.

Results

Clinical course

Six paediatric patients with primary hepatocellular carcinoma were treated with immunotherapy between June 2015–August 2019. Patient characteristics, prior treatment, response, toxicities, and life status can be found in Table 1. *Patient 1,* a then 17-year-old male with metastatic, multiply recurrent fibrolamellar carcinoma (FLC), initiated treatment with single-agent pembrolizumab upon development of a biopsy-proven right hilar mass and progression of biopsy-proven lung nodules. He received concurrent radiotherapy to a dose of 4800 cGy to the right hilar mass. After three months of therapy, he achieved a PR at the right hilar mass and shrinkage of the existing pulmonary nodules. After six months of pembrolizumab therapy, he achieved a CR of the remaining pulmonary nodules (Fig. 1a/b). Six months into therapy he developed a persistent elevation in liver function studies (Grade 3) prompting administration of pembrolizumab every 4 weeks as opposed to every 3 weeks. Six months later, he developed mild increased work of breathing and intermittent cough and was found on chest imaging to have evidence consistent with an immune-mediated pneumonitis (Grade 2). Pembrolizumab was held and he initiated a steroid course with protracted taper. Symptoms recurred with a pembrolizumab re-challenge post-taper therefore the drug was discontinued after receipt of 25 total cycles. He remained in CR for 18 months and ultimately went on to require liver transplantation for progressive liver dysfunction perceived secondary to chronic liver ischemia sustained from upfront ex-vivo resection.[19] He remains in remission 2 years post-transplant.

Patient 2, a then 14-year-old male with recurrent conventional hepatocellular carcinoma (HCC), initiated treatment with pembrolizumab upon imaging evidence of progressive lung nodules and elevation in serum AFP. He received 15 cycles of pembrolizumab during which his AFP remained stable and imaging confirmed SD. Growth of a prominent lung nodule following these 15 cycles prompted radiofrequency ablation (RFA) to the lesion, pivot to 2 cycles of nivolumab/ipilimumab, and eventual treatment with a first-in-human single-patient investigational new drug protocol for use of a synthetic peptide vaccine (Fig. 1c). He received 11 doses of vaccine during which time his AFP rose more sharply and he developed hypothyroidism (Grade 2) requiring levothyroxine supplementation. Approval from the Food and Drug Administration was obtained to administer pembrolizumab concurrent with vaccine but shortly following administration of the combination, the patient was noted to have a new scapular bony metastasis and subsequently a lesion in his pubic ramus both of which underwent cryotherapy and subsequently treatment with external beam radiotherapy. Rises in AFP correlated with new sites of rapid growth (scapula, pelvis, intra-abdominal lymph node) but his lung disease continued to demonstrate stability for greater than 1 year (Fig. 1d). In the seven years following initiation of single-agent pembrolizumab, the patient received treatment with various forms of immunotherapy paired with surgical or interventional procedures (pembrolizumab/bevacizumab, pembrolizumab/2nd generation vaccine, engineered T-cells targeting AFP and glypican-3). He died of disease 8 years following his initial diagnosis.

Patient 3, a then 15-year-old female with recurrent metastatic FLC, initiated pembrolizumab after development of new lung metastasis following upfront primary tumour resection. She achieved SD during receipt of 14 cycles of pembrolizumab; these were well-tolerated apart from an intermittently elevated lipase (Grade 1) and loose stools (Grade 1). Growth of a right lower lobe lung nodule prompted ablation but shortly thereafter she developed a new mediastinal site of disease. This site received 4000 cGy of radiotherapy and she went on to receive an additional 35 cycles of pembrolizumab. Ultimately, the patient experienced progression of mediastinal disease despite a switch to nivolumab/ipilimumab and receipt of additional radiotherapy. She succumbed to disease 6 years following her diagnosis.

Patients 4–6 experienced more rapidly progressive disease despite treatment with immunotherapy. *Patient 4,* a then 16-year-old male with recurrent FLC in the liver following resection of the primary tumour, received 10 cycles of pembrolizumab with Yttrium-90 delivered to the primary site of disease. The treatment was well tolerated with no side effects but he developed progression of his primary liver tumour with development of lung metastases while receiving this therapy. He experienced rapid progression of disease thereafter and ultimately died 2 years following disease recurrence. *Patient 5,* a then 12-year-old male with recurrent FLC in the liver, received 7 cycles of pembrolizumab before developing progression in the liver and peritoneum. He experienced mild elevation of liver enzymes (Grade 1) and loose stools (Grade 2). He too experienced

Variable	Value
Age at diagnosis (median, IQR, Q1, Q3 in years)	13 (2, 12, 13)
Sex (n)	
Male	5
Female	1
Age at receipt of immunotherapy (median, IQR, Q1, Q3 in years)	15.5 (2, 14.5, 16)
Histology (n)	
Conventional	1
Fibrolamellar	5
Localized disease (n)	0
Metastatic disease (n)	6
Receipt of chemotherapy prior to immunotherapy (n)	4
Type of immunotherapy (n)	
Checkpoint inhibitor monotherapy	6
Addition of radiotherapy	1
Addition of personalized vaccine	1
Addition of interventional procedures (ablation, cryotherapy, Yttrium-90)	3
Dual checkpoint inhibitor therapy	3
Addition of radiotherapy	1
Toxicities (graded per CTCAE 5.0)	
Transaminitis (Grade 1, Grade 3)	2
Pneumonitis (Grade 2)	1
Hypothyroidism (Grade 2)	1
Elevated lipase (Grade 1)	1
Loose stools (Grade 1, Grade 2)	2
Overall response (n)	
CR	1
SD	2
PD	3
Life status (n)	
Alive in remission	1
Dead of disease	5
Oncopanel pre-immunotherapy	5
Immunohistochemistry pre-immunotherapy	5
Nanostring on pre-treatment tissue	5
Nanostring on serial tissue samples	5

IQR: Interquartile range.

Table 1: **Patient, treatment characteristics, and available samples for associational analyses.**

rapid progression of disease and died 2 years following diagnosis. Finally, *Patient 6*, a then 16-year-old male with FLC recurrent to the liver, received 2 cycles of pembrolizumab and one cycle of nivolumab/ipilimumab which were well tolerated but resulted in disease growth. He achieved disease stability on an oral anti-angiogenic regimen for nearly 10 months before undergoing a multivisceral transplant. He recurred shortly thereafter and died approximately 3 years following his initial recurrence. Fig. 2 depicts the timeline for receipt of immunotherapy for each of the patients described above. Additional information regarding treatment course can be found in Supplemental Table S1.

Tumour genomic profiling

The mean tumour mutational burden for this patient series was 4.5 mutations/Mb (median: 4.2, range: 2.4–8.4). *Patient 1*, who achieved a complete response, had a tumour mutational burden of 8.4 mutations/Mb. The definition of "high" tumour mutational burden in most adult studies is ≥ 10 mutations/Mb.[20] Only two patients had mutations deemed clinically relevant or actionable on analysis of Oncopanel results. *Patient 2* had both a somatic *CTNNB1* base pair deletion and a *MAPK1* mutation (Table 2). The observed deletion in *CTNNB1* includes exon3, the site of phosphorylation, and leaves intact the functional Armadillo domain. This alteration is predicted to result in activation of the Wnt pathway via alteration of the phosphorylation sites, which leads to constitutive persistance of β-catenin and translocation to the nucleus in the absence of upstream signaling. This predicted upregulation is supported by nuclear beta-catenin expression by immunohistochemistry in this patient's tumour.[21] *Patient 4* had a homozygous mutation in the *MUTYH* gene indicative of a carrier state for heritable *MUTYH*-associated polyposis syndrome. There are no published associations between fibrolamellar carcinoma and *MUTYH*-associated polyposis. Of note, *Patient 1* had variants of uncertain significance identified in both *NF1* and *PIK3C2B*; somatic mutations in *NF1* have been reported in conventional HCC but not fibrolamellar carcinoma.[22] *Patient 6* had a *mTOR* mutation of uncertain significance; of note, *mTOR* activating mutations have been described in association with fibrolamellar carcinoma.[23] Increased copy number variation (CNV) has been linked to poor immunotherapy response; in keeping, *Patient 6* demonstrated a higher rate of copy number variation that the other patients in this series.[24]

Immunophenotype

Immunohistochemistry

Immunohistochemistry was performed on diagnostic tissue, when available, or post-treatment specimens to assess for intratumoural infiltrating immune cells. The type of specimen, timing (pre-immunotherapy highlighted in orange) and staining characteristics are listed in Supplemental Table S2. Lung tissue demonstrated a trend towards significance in the number of tallied CD45+ cells when compared to liver ($p = 0.13$, Wilcoxon rank sum) and lymph/soft tissue ($p = 0.11$, Wilcoxon rank sum). When analyzing this data utilizing a comparison of the means with report of 95% confidence intervals, the results were as follows: lung vs. liver (443, 95% CI: −78 to 946) and lung vs. lymph/soft tissue (431, 95% CI: −292 to 1152) indicating a less convincing trend towards significance likely due to small sample size. There were notable observations among the three patients that sustained a response (CR or SD) while receiving immunotherapy. A lung nodule sampled from *Patient 1* prior to the start of

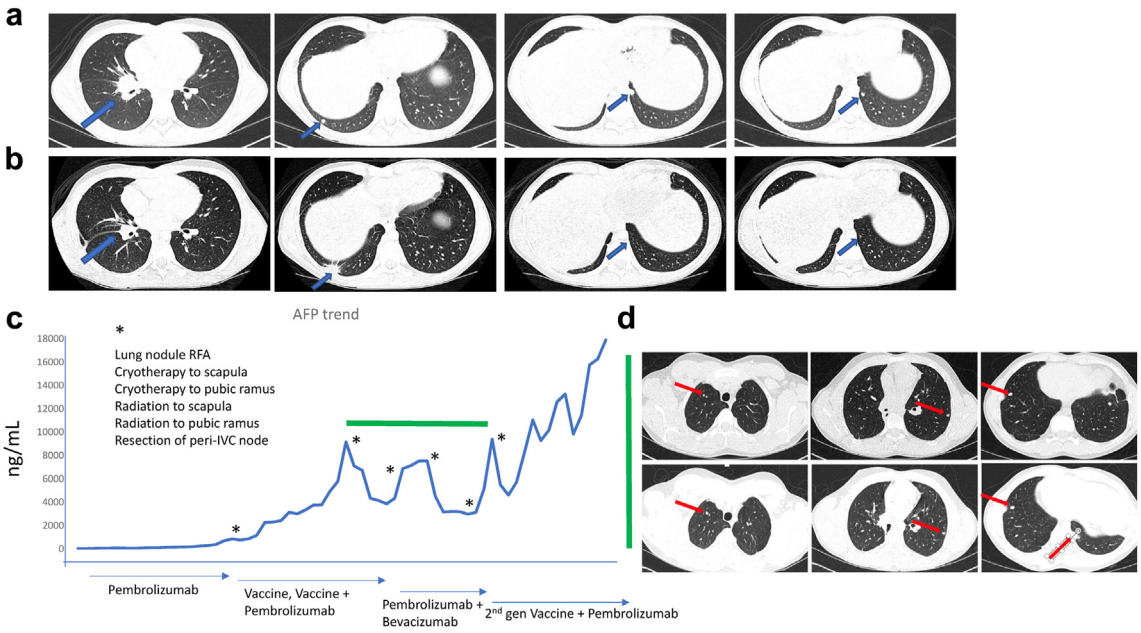

Fig. 1: Panel a (*Patient 1*): Right hilar lung lesion and multiple pulmonary nodules prior to initiation of checkpoint inhibitor therapy and external beam radiotherapy. Panel b (*Patient 1*): Diminished size of right hilar mass following radiotherapy. Post-thoracotomy changes at site of lung nodule sampling (second panel) and complete resolution of residual lung lesions (third and fourth panels). Panel c (*Patient 2*): Prolonged period of AFP stability following initiation of single-agent checkpoint inhibition. Asterisks signifying local control interventions to address new sites of disease growth correlating with AFP rise. Panel d (*Patient 2*): Lung lesion stability over the course of 12 months (top row baseline, bottom row 12 months later). This period of lung nodule stability overlaps in time with the growth of new sites of disease (scapula, pubic ramus, peri-IVC) as indicated by the green line.

immunotherapy demonstrated a prominent CD45+ infiltrate (73% of cells being CD3+) as well as PD-1 and PDL-1 staining. A lung nodule sampled at the time of post-chemotherapy relapse and prior to the start of immunotherapy in *Patient 2* demonstrated a moderate CD45+ infiltrate (16% CD3+) and PD-1 expression. Both liver and lung specimens from diagnosis, pre-immunotherapy, showed a moderate and prominent CD45+ (60–70% CD3+) infiltrate, respectively, with associated PD-1 expression. For *Patients 2 and 3*, in whom serial samples were available following initiation of immunotherapy, PD-1 expression waned. There was no pre-immunotherapy specimen available for *Patient 4* and while a moderate CD45+ count was

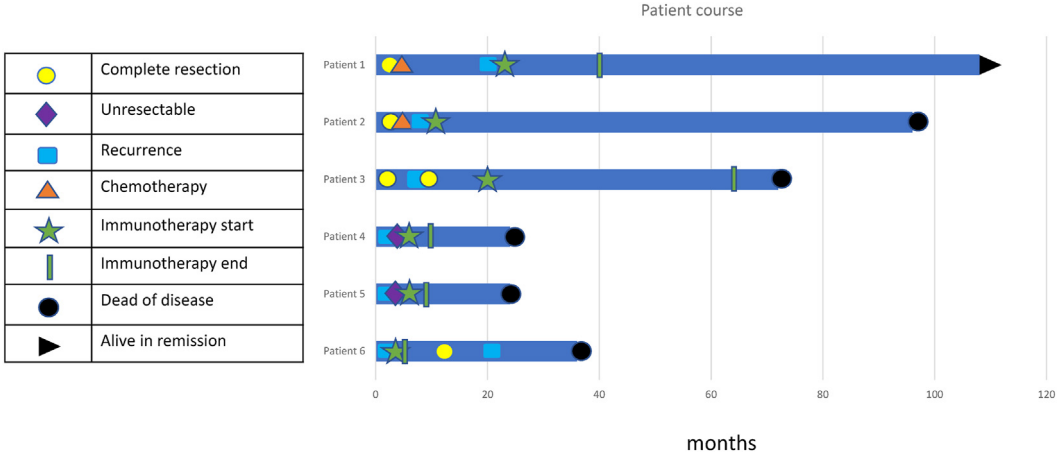

Fig. 2: Swimmers plots for each individual patient course.

Patient	Specimen	TMB (mut/Mb)	*DNAJB1::PRKACA* fusion	Clinically significant variants[a]	Variants of uncertain significance[b]	Selected copy number alterations (+gain, –loss)
1	Soft tissue, pre-peritoneal, s/p chemotherapy, pre-immunotherapy	8.4	Present	None	*PIK3C2B* p.R564L *MDM4* p.I175T *CBLB* p.G128N *PDGFRA* p.T276M *SF1* p.A410S *KMT2A* p.A53V *KMT2D* p.R2188L *NFKBIA* p.L25Q *NF1* p.L532M *SETBP1* p.T1260M *JAK3* p.V722I	+ *FGFR3* +8q –10q –21q
2	Lung, Diagnostic bx, pre-immunotherapy	2.4	Absent	*CTNNB1* c.13 + 10_432del[†] *MAPK1* p.E322K	*PRKDC* p.A3391V	–1 –2q +20q
3	Lymph node, recurrence, post-immunotherapy	4.6	Present	None	*TERT* c.-355_-354del (5′UTR) *CEBPA* p.E148K *DCLRE1C* p.G185E *ERCC6* p.R479C *ERCC6* p.G36D *FANCE* p.R92W *OGG1* c.565+2T > A *PTCH1* p.K746Q *SDHA* p.V632F	+1p –8p +8q –18q
4	Liver, at recurrence, pre-immunotherapy	5.3	Present	*MUYTH* p.Y101	*BRE* p.R272K *BRIP1* p.A185T *CIC* p.K94R *COL7A1* p.V1973M *CUX1* p.N261S *RECQL4* p.E216G *RINT* p.M342T	+8 +15q –18q
5	Liver, primary resection, pre-immunotherapy	2.4	Present	None	*ADAM6* c.45134_splice *EPHA7* p.1556V *ERCC3* p.R742W	+5p –14q +16q
6	Liver, s/p 1 cycle of chemotherapy, pre-immunotherapy	3.8	Present	None	*DEPDC5* p.G17V *MTOR* p.Q2499R *PMS2* c.354-1G > A	Gains and losses throughout the targeted genome

[a]Tiers 1 and 2 according to AMP/CAP/ASCO guideline. [b]Tier 3 according to AMP/CAP/ASCO guideline.

Table 2: **Specimen, timing, and results to somatic tumour genomic profiling.**

detected for *Patients 5 and 6*, these patient samples did not stain for PD-1 expression.

Nanostring

Patients were grouped by best response into one of two categories: "responders" (*Patients 1–3*) or "non-responders" (*Patients 4–6*). Samples from all available time points were grouped into these two categories. Samples from "responders" demonstrated decreased expression of genes associated with T-cell dysfunction (*LAG3, CX3XL1, CD96, CD83, CD96*), immunosuppression and/or inflammation (*OAS3, CCL4, ENTPD1, CD36, CD163, TFRC, LIF*) and cancer growth and invasion (*MCAM, ICAM1, ICAM2*). Samples profiled from *Patient 1*, immediately following discontinuation of cytotoxic chemotherapy, demonstrated expression profiles suggestive of depleted immune function. Profiling of a sample collected years off chemotherapy and prior to immunotherapy clustered with "responder" samples from *Patients 2* and *3*. *Patient 3* had expression profiling of a lymph node sample obtained after numerous cycles of checkpoint inhibition; expression patterns demonstrated progressive evidence of T-cell dysfunction over time. *Patients 4 and* 5 had relatively quiet gene expression signatures. *Patient 6*, who experienced rapid progression of disease on immunotherapy, had markedly *upregulated* genes in the categories referenced above (Fig. 3a). The volcano plot demonstrates genes with the highest fold up- or down-regulation. Of note, the sample from *Patient 2* post-immunotherapy demonstrates overexpression of MAP2K1 perhaps reflective of the underlying somatic tumour mutation or an evolving immunosuppressive environment.

Discussion

This manuscript describes the treatment of a series of paediatric patients with primary liver carcinoma treated

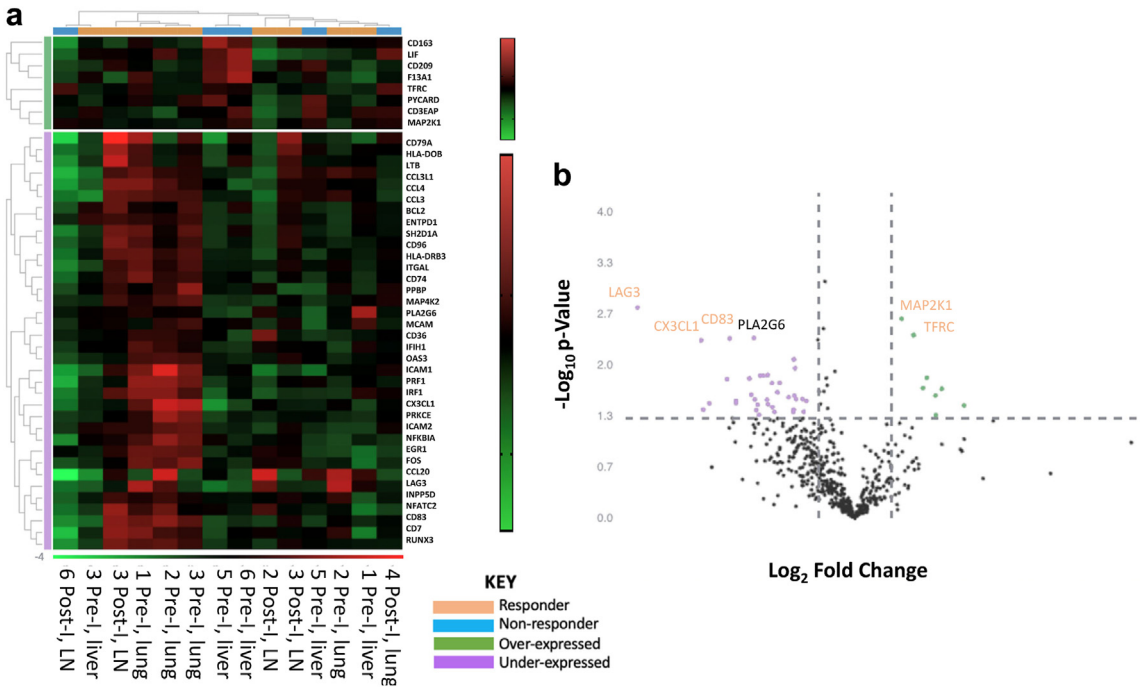

Fig. 3: Panel a: Heatmap demonstrating unsupervised clustering of gene expression profiles for patients in the treated cohort. Pre-immunotherapy specimens for *Patients* 1–3 cluster tightly and demonstrate down-regulation in expression of genes relevant to T-cell dysfunction, inflammation, and tumour growth and invasion. Panel b: Volcano plot indicating genes with highest-fold up- or down-regulation (highly over- or under-expressed genes, relevant to responders, are colour coded in orange, aligned with the colour code schematic utilized in the adjacent heatmap).

with immunotherapy with the goal to report on tolerability, efficacy, and correlative biology potentially predictive of response. We identify patients that respond to immunotherapy and preliminary clinical or genomic biomarkers correlating with response. Notably, we report on a complete response to single-agent checkpoint inhibition in a patient with multiply recurrent, metastatic fibrolamellar carcinoma—a typically incurable clinical scenario.

We found clinical and genomic features relevant to "responders" vs. "non-responders." First, "responders" tended to have a lower disease burden with disease confined to the lungs as opposed to bulky disease in the liver, lymph nodes, or peritoneum. Given the inherently immunosuppressive environment of the liver and accessibility of small lung lesions to immune cells, disease location and burden may favorably predict response to immunotherapy. Immunohistochemical stains confirmed expression of PD-1 or PD-L1 in "responders". Only two somatic genomic features correlated with response: a comparatively high tumour mutational burden in *Patient 1*, the "extraordinary responder", and a high copy number variation in *Patient 6*, the "poor responder" both of which are known to have positive and negative correlation, respectively, with immune response in adults.[24] "Responders" (i.e. *Patients 1–3)* were perhaps best predicted by bulk RNA-sequencing of serial tumour specimens which demonstrated decreased expression of genes associated with T-cell dysfunction, inflammation, and cancer growth and invasion. These conclusions are drawn with the acknowledgement of a small sample size and heterogeneous clinical characteristics and treatment approach.

There is an abundance of data predictive of response to immunotherapy in adult patients with cancer.[24,25] High tumour mutational burden and neoantigen load are among the biomarkers most closely linked; both are notoriously low in paediatric patient samples save for the findings in *Patient 1*. The same is true for tumours harboring deficiencies in DNA damage repair pathways none of which were detected in samples from this patient series. There are identified cancer predisposition syndromes in paediatric patients associated with aberrant DNA damage repair and predictive of enhanced response to immunotherapeutics.[26,27] We did not analyze germline genetics for these patients but no somatic variants were flagged as potentially clinically relevant with respect to germline predisposition.[28]

Dysregulation of the MAPK, PI3K-AKT-mTOR, and CTNNB1 pathways has been associated with decreased recruitment of T-cells and/or dysfunctional T-cells diminishing response to checkpoint blockade.[29]

Diagnostic tumour tissue from *Patient 2* had somatic mutations documented in both the CTNNB1 and MAPK pathways. Despite the emergence of possible escape clones prompting new sites of disease in the scapula, pubic ramus, and in peri-IVC region, this patient sustained prolonged stable disease in his lungs for over a year. External beam radiotherapy and cryotherapy have been postulated to expose novel neo-antigens capable of stimulating an abscopal effect supporting checkpoint blockade.[30] This mechanism may have been responsible for the responses noted in *Patients 1 and 2*. As might be expected, and in harmony with existing adult literature, downregulation of genes implicated in T-cell dysfunction and inflammation, noted for *Patients 1–3*, were likely among the greatest contributors to disease response and control.[24,25]

This study is undoubtedly limited by the retrospective nature of data collection, and the heterogeneity of disease histology, treatment approach, and specimen timepoints. It is likewise hindered by a small sample size and an associated low power limiting our ability to identify the associations as well as adjustment for confounders to the analysis. Despite this, we were able to detect signals of response in patients treated with both single-agent checkpoint inhibitor therapy and combination immunotherapy. Counter to the belief that paediatric disease does not respond to immunotherapy, these findings suggest that there are some patients who respond and some patients for whom tissue expression profiles may provide insights regarding response. These findings may have implications for additional paediatric carcinomas or diseases affecting the adolescent and young adult patient population. This patient series has informed a multi-institutional, national, funded trial to study the safety and efficacy of single-agent checkpoint inhibition in paediatric patients with relapsed/refractory primary liver carcinomas (NCT04134559). In light of trends in adult practice towards the use of dual checkpoint inhibition frontline for HCC, this trial will be amended to offer treatment with dual checkpoint inhibition to allow the prospective study of two agents, and correlates of response, in paediatric patients. The trial will continue to analyze serial circulating biomarkers including, but not limited to, circulating immune cells, cell-free DNA, and cytokines. Following administration of three cycles of therapy, the trial also allows introduction of external beam radiotherapy or interventional procedures intended to study the abscopal effect.

The paediatric community has likewise engaged in the study of antibody-based and engineered T-cell therapies (NCT04928677, NCT04377932, NCT04634357) for this difficult-to-treat population with relapsed/refractory primary liver tumours. Each of these trials intends to serially study immune biomarkers correlating with efficacy. The Children's Oncology Group protocol, AHEP1531, conducted internationally with consortia in Europe and Japan, included a dedicated arm for the study of paediatric patients with HCC for the first time in history. This trial recently closed to accrual and work focused on design of an international successor trial is currently underway. Results to NCT04134559 will undoubtedly guide whether immunotherapy should be studied prospectively in a larger patient cohort. This portfolio of trials may further elucidate which disease characteristics or genomic findings best predict response. Similarly, our results support further exploration of the role of immunotherapy in choice paediatric diseases and promote further in-depth study of tissue or serum correlatives to identify patients most apt to benefit from novel immunotherapeutics in the future.

Contributors

Conceptualization, data curation, formal analysis, investigation, methodology, project administration, and resources: AF O'Neill, AJ Church, C Rodrgieuz-Galindo.

Funding acquisition: AF O'Neill.

Drafting of the article, supervision, and validation: AF O'Neill, AJ Church, A Feraco, J Spidle, CB Wall, HB Kim, S Elisofon, K Vakili, M Pimkin, NV Dharia, NR Shelman, AR Perez-Atayde, C Rodrgieuz-Galindo.

Verification of data: AF O'Neill, AJ Church, NR Shelman, AR Perez-Atayde, C Rodriguez-Galindo.

Final approval of the version to be published: AF O'Neill, AJ Church, A Feraco, J Spidle, CB Wall, HB Kim, S Elisofon, K Vakili, M Pimkin, NV Dharia, NR Shelman, AR Perez-Atayde, C Rodrgieuz-Galindo.

Data sharing statement

Granular data generated with use of Nanostring is available upon author request. The remainder of the data is presented in its original form in the manuscript.

Declaration of interests

All authors report no conflicts of interest relevant to this article. A.C. has received grants or contracts from the Children's Oncology Group, the National Cancer Institute and the American Academy of Cancer Research; she is likewise a consultant for Jackson Laboratories and has received speaker fees from Tecan. N.V.D. and M.P were former employees of Dana-Farber Cancer Institute when the patients described were cared for; N.V.D is now an employee of Genentech, a member of the Roche Group where he has a patent pending. N.V.D. was receiving funding from Julia's Legacy of Hope Street and the Baldrick's Foundation Fellowship, unrelated to this work, but during the time period during which this work was being performed. M.P. is now an employee of Takeda. K.V. has a patent pending for a liver cancer therapeutic agent and stock options with ZEsST Bio.

Acknowledgements

This work was supported by philanthropic funds (Pan Mass Challenge, Team Angus and Team Perspective).

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2024.105147.

References

1 Bulterys MGM, Smith MA, Buckley JD. SEER pediatric monograph. In: *Chapter VII Hepatic Tumors 91–99*. 1975-1995.
2 Khanna R, Verma SK. Pediatric hepatocellular carcinoma. *World J Gastroenterol*. 2018;24:3980–3999.
3 Haines K, Sarabia SF, Alvarez KR, et al. Characterization of pediatric hepatocellular carcinoma reveals genomic heterogeneity and diverse signaling pathway activation. *Pediatr Blood Cancer*. 2019;66:e27745.

4 Lopez-Terrada D, Alaggio R, de Davila MT, et al. Towards an international pediatric liver tumor consensus classification: proceedings of the Los Angeles COG liver tumors symposium. *Mod Pathol*. 2014;27:472–491.
5 O'Neill AF, Meyers RL, Katzenstein HM, et al. Children's Oncology Group's 2023 blueprint for research: pediatric liver tumors. *Pediatr Blood Cancer*. 2023;6:e30576.
6 Katzenstein HM, Krailo MD, Malogolowkin MH, et al. Hepatocellular carcinoma in children and adolescents: results from the Pediatric Oncology Group and the Children's Cancer Group intergroup study. *J Clin Oncol*. 2002;20:2789–2797.
7 Schmid I, von Schweinitz D. Pediatric hepatocellular carcinoma: challenges and solutions. *J Hepatocell Carcinoma*. 2017;4:15–21.
8 Schmid I, Haberle B, Albert MH, et al. Sorafenib and cisplatin/doxorubicin (PLADO) in pediatric hepatocellular carcinoma. *Pediatr Blood Cancer*. 2012;58:539–544.
9 O'Neill AF, Church AJ, Perez-Atayde AR, et al. Fibrolamellar carcinoma: an entity all its own. *Curr Probl Cancer*. 2021;45:100770.
10 Salem R, Li D, Sommer N, et al. Characterization of response to atezolizumab + bevacizumab versus sorafenib for hepatocellular carcinoma: results from the IMbrave150 trial. *Cancer Med*. 2021;10:5437–5447.
11 Geoerger B, Kang HJ, Yalon-Oren M, et al. Pembrolizumab in paediatric patients with advanced melanoma or a PD-L1-positive, advanced, relapsed, or refractory solid tumour or lymphoma (KEYNOTE-051): interim analysis of an open-label, single-arm, phase 1-2 trial. *Lancet Oncol*. 2020;21:121–133.
12 Wheeler DA, Wang L. From human genome to cancer genome: the first decade. *Genome Res*. 2013;23:1054–1062.
13 Church AJ, Corson LB, Kao PC, et al. Molecular profiling identifies targeted therapy opportunities in pediatric solid cancer. *Nat Med*. 2022;28:1581–1589.
14 Garcia EP, Minkovsky A, Jia Y, et al. Validation of OncoPanel: a targeted next-generation sequencing assay for the detection of somatic variants in cancer. *Arch Pathol Lab Med*. 2017;141:751–758.
15 Sholl LM, Do K, Shivdasani P, et al. Institutional implementation of clinical tumor profiling on an unselected cancer population. *JCI Insight*. 2016;1:e87062.
16 Li MM, Datto M, Duncavage EJ, et al. Standards and guidelines for the interpretation and reporting of sequence variants in cancer: a joint consensus recommendation of the association for molecular Pathology, American society of clinical Oncology, and College of American Pathologists. *J Mol Diagn*. 2017;19:4–23.
17 Al-Ibraheemi A, Putra J, Tsai HK, et al. Assessment of BCOR internal tandem duplications in pediatric cancers by targeted RNA sequencing. *J Mol Diagn*. 2021;23:1269–1278.
18 Brahmer JR, Lacchetti C, Schneider BJ, et al. Management of immune-related adverse events in patients treated with immune checkpoint inhibitor therapy: American society of clinical Oncology clinical practice guideline. *J Clin Oncol*. 2018;36:1714–1768.
19 LaQuaglia MJKH, Fynn-Thompson F, Baird C, Vakili K. Resection of hepatic tumors with central venous and right atrial extension using cardiopulmonary bypass. *J Pediatr Surg Case Rep*. 2018;30:14–18.
20 Strickler JH, Hanks BA, Khasraw M. Tumor mutational burden as a predictor of immunotherapy response: is more always better? *Clin Cancer Res*. 2021;27:1236–1241.
21 Lee SE, Chang SH, Kim WY, et al. Frequent somatic TERT promoter mutations and CTNNB1 mutations in hepatocellular carcinoma. *Oncotarget*. 2016;7:69267–69275.
22 Kaseb AO, Sanchez NS, Sen S, et al. Molecular profiling of hepatocellular carcinoma using circulating cell-free DNA. *Clin Cancer Res*. 2019;25:6107–6118.
23 Sahin F, Kannangai R, Adegbola O, et al. mTOR and P70 S6 kinase expression in primary liver neoplasms. *Clin Cancer Res*. 2004;10:8421–8425.
24 Cormedi MCV, Van Allen EM, Colli LM. Predicting immunotherapy response through genomics. *Curr Opin Genet Dev*. 2021;66:1–9.
25 Charoentong P, Finotello F, Angelova M, et al. Pan-cancer immunogenomic analyses reveal genotype-immunophenotype relationships and predictors of response to checkpoint blockade. *Cell Rep*. 2017;18:248–262.
26 Das A, Sudhaman S, Morgenstern D, et al. Genomic predictors of response to PD-1 inhibition in children with germline DNA replication repair deficiency. *Nat Med*. 2022;28:125–135.
27 Tan MH, Mester JL, Ngeow J, et al. Lifetime cancer risks in individuals with germline PTEN mutations. *Clin Cancer Res*. 2012;18:400–407.
28 Schienda J, Church AJ, Corson LB, et al. Germline sequencing improves tumor-only sequencing interpretation in a precision genomic study of patients with pediatric solid tumor. *JCO Precis Oncol*. 2021;5:PO.21.00281. https://doi.org/10.1200/PO.21.00281.
29 Yang L, Li A, Lei Q, et al. Tumor-intrinsic signaling pathways: key roles in the regulation of the immunosuppressive tumor microenvironment. *J Hematol Oncol*. 2019;12:125.
30 Postow MA, Callahan MK, Barker CA, et al. Immunologic correlates of the abscopal effect in a patient with melanoma. *N Engl J Med*. 2012;366:925–931.