Clinical response to nivolumab in an INI1-deficient pediatric chordoma correlates with immunogenic recognition of brachyury

Laura M. Williamson1, Craig M. Rive2, Daniela Di Francesco1, Emma Titmuss2, Hye-Jung E. Chun1, Scott D. Brown1, Katy Milne2,3, Erin Pleasance1, Anna F. Lee2, Stephen Yip2, Daniel G. Rosenbaum6, Martin Hasselblatt2, Pascal D. Johann1,15, Marcel Kool8,9,11,12, Rebecca J. Deyell12 and Marco A. Marra11,15.

Poorly differentiated chordoma (PDC) is a recently recognized subtype of chordoma characterized by expression of the embryonic transcription factor, brachyury, and loss of INI1. PDC primarily affects children and is associated with a poor prognosis and limited treatment options. Here we describe the molecular and immune tumour microenvironment profiles of two paediatric PDCs produced using whole-genome, transcriptome and whole-genome bisulphite sequencing (WGBS) and multiplex immunohistochemistry. Our analyses revealed the presence of tumour-associated immune cells, including CD8+ T cells, and expression of the immune checkpoint protein, PD-L1, in both patient samples. Molecular profiling provided the rationale for immune checkpoint inhibitor (ICI) therapy, which resulted in a clinical and radiographic response. A dominant T cell receptor (TCR) clone specific for a brachyury peptide–MHC complex was identified from bulk RNA sequencing, suggesting that targeting of the brachyury tumour antigen by tumour-associated T cells may underlie this clinical response to ICI. Correlative analysis with rhabdoid tumours, another INI1-deficient paediatric malignancy, suggests that a subset of tumours may share common immune phenotypes, indicating the potential for a therapeutically targetable subgroup of challenging paediatric cancers.

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INTRODUCTION

Chordomas are malignant neoplasms derived from notochord cells typically originating in the spine, sacrum or base of the skull, affecting both adults and children. Poorly differentiated chordomas (PDCs) are a recently recognized distinct subgroup of chordoma, which occurs predominantly in children, and is characterized by the expression of brachyury, an embryonic transcription factor encoded by the TBXT gene, and by loss of the SWI/SNF chromatin remodelling factor subunit, INI1, encoded by the SMARCB1 gene1–3. These aggressive tumours are associated with a high risk of rapid local recurrence and distant metastasis and are generally poor candidates for primary resection, highlighting the need for effective systemic therapy.

Immune checkpoint inhibitors (ICIs) are now the standard of care for various adult malignancies associated with high tumour mutation burden (TMB) including lung cancer, melanoma and tumours deficient in mismatch repair4. Paediatric tumours are typically associated with low TMB compared to adult tumours, and emerging data from several prospective clinical trials have reported an overall low rate of response to ICIs in solid paediatric tumours5–7. Although broad response to ICIs among paediatric patients is lacking, growing evidence has shown responses to ICIs in tumours deficient in SWI/SNF chromatin remodelling genes, including but not limited to chordomas, rhabdoid tumours (RTs), and small cell carcinoma of the ovary hypercalcemic type tumours that affect paediatric and young adult populations5–10. This highlights the need to identify those patients most likely to benefit from ICIs and distinguish these from the broader paediatric population. The presence of infiltrating CD8+ T cells and expression of immune checkpoint genes in a subset of tumours is compatible with the notion that malignancies characterized by SWI/SNF deficiency may correlate with an immune hot phenotype and therapeutic response to ICIs10,11,13,14,16,17.

Here we describe the molecular and immune tumour microenvironment profiles of two paediatric PDCs using whole-genome, transcriptome and whole-genome bisulphite sequencing (WGBS) and multiplex immunohistochemistry (IHC) as part of the Personalized OncoGenomics (POG) programme (NCT02155621) at BC Cancer. Our analyses provided evidence for the presence of tumour-infiltrating CD8+ T cells, a brachyury tumour antigen-directed immune response and the rationale for ICI therapy that...
resulted in a clinical and radiographic response in a PDC patient treated with nivolumab. Analysis of methylation profiles from paediatric chordomas and SWI/SNF-deficient RTs revealed a correlation between paediatric chordoma and a subgroup of RTs characterized by CD8+ T cell infiltration, supporting the hypothesis that molecularly similar entities from different cancer types may also benefit from ICI therapy.

RESULTS
Clinical presentation
Patient 1 presented with worsening stiffness and pain of the neck. A magnetic resonance imaging (MRI) showed an infiltrating but non-enhancing soft tissue mass around the anterior atlantoaxial joint causing erosion of the bone and extending from the clivus into the prevertebral space to the right of C2. A computed tomographic (CT) scan showed bony erosion of both C1 and C2 and subsequent positron emission tomographic (PET) imaging revealed a fluorodeoxyglucose (FDG)-avid soft tissue mass of the neck (SUVmax 7.15) and left hilar adenopathy (SUVmax 2.34). Biopsy of the primary mass revealed an INI1-negative spindle cell neoplasm. A second biopsy was consistent with a diagnosis of an epithelioid sarcoma, with morphology more consistent with the conventional rather than the proximal type, with absence of overt rhabdoid morphology. Positive nuclear brachyury staining, strong keratin staining and negativity for S100 and INI1 supported a diagnosis of PDC (Fig. 1a, c, d and Supplementary Fig. 1). Patient 1 was treated with neoadjuvant vincristine, doxorubicin and cyclophosphamide with alternating ifosfamide and etoposide with good response, followed by high-dose proton beam radiation. The patient recurred with metastatic disease of the lung 3 months following radiation, and it was this metastatic site that was biopsied for whole-genome and transcriptome analysis (WGTA). Three additional cycles of chemotherapy were given with mixed response, and the patient died within a month of progressive disease.

Patient 2 presented with a 3-year history of recurrent headaches, followed by worsening neck discomfort and limited range of movement. An MRI of her head and neck revealed a large, multi-lobulated expansile soft tissue mass centred at the left anterior arch and lateral mass of the C1 vertebral body, measuring 4.3 × 2.1 × 2.8 cm and involving the clivus, occipital condyle and dens of C2. There was mass effect on the adjacent left vertebral artery and right anterior medulla, as well as on the tectorial membrane and para-pharyngeal space. Chest CT showed two irregular pleural-based densities that were resected and consistent with metastatic disease. A PET scan showed an FDG-avid mass at C1 involving the lateral mass, the dens, the clivus and the left occipital condyle. Two faintly avid densities were noted in the right lung, with lower PET avidity than would be expected of metastases. No other distant metastases were seen. A biopsy of the C1 mass was composed of sheets of epithelioid cells. Tumour cells were immunonegative for S100 and subsequent positron emission tomographic (PET) imaging revealed a fluorodeoxyglucose (FDG)-avid soft tissue mass of the neck (SUVmax 7.15) and left hilar adenopathy (SUVmax 2.34). Biopsy of the primary mass revealed an INI1-negative spindle cell neoplasm. A second biopsy was consistent with a diagnosis of an epithelioid sarcoma, with morphology more consistent with the conventional rather than the proximal type, with absence of overt rhabdoid morphology. Positive nuclear brachyury staining, strong keratin staining and negativity for S100 and INI1 supported a diagnosis of PDC (Fig. 1a, c, d and Supplementary Fig. 1). Patient 1 was treated with neoadjuvant vincristine, doxorubicin and cyclophosphamide with alternating ifosfamide and etoposide with good response, followed by high-dose proton beam radiation. The patient recurred with metastatic disease of the lung 3 months following radiation, and it was this metastatic site that was biopsied for whole-genome and transcriptome analysis (WGTA). Three additional cycles of chemotherapy were given with mixed response, and the patient died within a month of progressive disease.

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Clinical response to nivolumab
While hypermutated tumours have been associated with response to ICIs22,28,29, paediatric cancers typically exhibit low TMB. Despite a low mutation burden as measured from whole-genome sequencing, estimates of CD8+ T cell abundance provided a rationale for ICI therapy3,22. Unfortunately, patient 1 died shortly after tumour sequencing and was not treated with

Genomic and transcriptomic analysis of poorly differentiated paediatric chordomas
WGTA detected low somatic TMBs for both cases (1.83 and 1.75 mutations per megabase for patients 1 and 2, respectively). Notable alterations in both cases included single copy losses affecting SMARCB1 (Fig. 1g), that were associated with low expression of SMARCB1 compared to both The Cancer Genome Atlas (TCGA) sarcoma18 data set and a pan-cancer cohort of adult cancers, referred to as POG570, sequenced as part of the POG programme19 (Fig. 1h and Supplementary Table 1). In patient 1’s tumour, we detected apparent biallelic loss of SMARCB1 as a result of an inverted translocation between SMARCB1 intron 3 and a non-coding region on chromosome 5 (hg19 chr22:24138567–chr5:176754524). Consistent with diagnostic IHC testing, the TBX7 gene that encodes the transcription factor brachyury was highly expressed in both samples compared to the TCGA sarcoma and POG570 data sets (Fig. 1h). WGBS data revealed hypomethylation of the TBX7 promoter (~1800 to ~3000 base pairs relative to the transcription start site), compatible with the notion that loss of TBX7 promoter DNA methylation may drive increased gene expression in these chordomas (Fig. 1i).

Immune markers associated with paediatric chordoma
Previous studies indicated linkages between SWI/SNF mutations and tumour immunogenicity.14,15,20, We explored the immune microenvironment of poorly differentiated SMARCB1-deficient chordomas using CIBERSORT21 to estimate the relative abundance of immune cell types from RNA sequencing (RNA-seq) data. Gene expression-based estimates of immune cell abundance have the potential to identify patients that may respond to ICIs across multiple cancer types, including sarcomas22,23. Our analysis revealed that both chordomas exhibited high CD8+ T cell expression signatures compared to several other paediatric and adult cancer data sets, including paediatric cranial and extracranial RTs that are similarly SMARCB1/INI1-deficient24,25 (Fig. 2a and Supplementary Fig. 2). Plasma and B cell abundance estimates were similarly high in the paediatric chordomas, particularly for patient 2, indicating a heterogenous population of lymphocytes present in the tumour microenvironment (Fig. 2a).

To confirm and further explore the CIBERSORT-predicted high CD8+ T cell expression signatures, tumour biopsy samples were analysed using multiplex IHC staining. Staining with a T cell panel confirmed the presence of CD3+CD8− and CD3+CD8+ T cells, including granzyme B (GZMB)+CD3+CD8+ T cells in both samples (Fig. 2b, c). Gene expression analysis revealed high expression of CD274 (programmed cell death ligand 1 (PD-L1)) compared to TCGA sarcoma (patient 1: 89th percentile, patient 2: 97th percentile) and POG570 pan-cancer data sets (patient 1: 83rd percentile, patient 2: 92nd percentile), indicating the presence of PD-L1-mediated immune evasion. In agreement with RNA expression data, IHC staining revealed PD-L1+positive cells in both tumour samples, with higher expression in patient 2 compared to patient 1. PD-L1 was expressed by both tumour and non-tumour cells as evidenced by the presence of PD-L1+/TBX7+ tumour cells in patient 2 and PD-L1+/CD68+ macrophages in patients 1 and 2 (Fig. 2c). Both samples also harboured CD79a+ B and plasma cells, which have been associated with ICI response in other cancers6,20,27.
immunotherapy or other molecularly targeted agents. The molecular tumour board recommended nivolumab, an anti-PD1 ICI with paediatric safety data, for patient 2 as a result of the evidence of a T cell-inflamed tumour. Given the lack of a suitable clinical trial, nivolumab was obtained via compassionate access following the failure of front line therapy. Following initiation of nivolumab, the patient experienced clinical improvement in symptoms, including decreased pain and improved mobility. MRI and PET/CT following three cycles of nivolumab showed partial response by Response Assessment in Neuro-Oncology (RANO) criteria, with an overall decrease in size (58% reduction in target lesion) and mass effect on the adjacent medulla (Fig. 3a, b). This partial response corresponded with a decrease in FDG-avidity (SUVmax 8.2 at baseline compared to 6.0 after 3 cycles). Although target lesion size remained smaller after 7 and 11 cycles compared to baseline, follow-up MRIs showed slow progression over time compared to documented best response. The patient remained on nivolumab for 14 cycles enabled by ongoing compassionate access to therapy, and continued to experience clinical benefit with an excellent functional status and quality of life.

Brachyury elicits patient-derived T cell receptor (TCR) activation

Given the clinical and radiographic response to nivolumab, we surveyed the RNA-seq data from patient 2 for evidence of tumour-associated T cells that may have mediated an anti-tumour immune response. Extraction of TCR-containing reads using MiXCR revealed 134 TCR alpha chain (TRA) and 110 beta chain (TRB) aligned reads corresponding to 91 and 68 distinct clones, respectively (Supplementary Table 2). The most dominant TRB clone, CSASTGRGTEAFF, was supported by 11.8% of TRB aligned reads, while the two most dominant TRA clones were supported by 6.0% (TRA-1, CAVQSNYKL5F) and 3.7% (TRA-2, CALGAVYMDTVAGLYTDKLIF) of TRA aligned reads (Fig. 4a). To assess the relative dominance of the major TCR sequence identified in this sample, we compared the frequencies of the most dominant TRB clone from patient 2 to the POG570 cohort. The relative TRB dominance for patient 2 was >63% of POG570 dominant clones, indicating a relatively abundant TCR clone in the tumour microenvironment (Supplemental Fig. 2b). These findings derived from RNA-seq data indicate the presence of a predominant, clonally expanded T cell population in the tumour microenvironment.

Brachyury protein expression is normally restricted to the developing embryo but can be aberrantly expressed in tumours and elicit an immune response. We speculated that the response of patient 2 to nivolumab may have been mediated by a brachyury tumour antigen. Peptide–major histocompatibility complex (MHC) (pMHC) binding predictions of brachyury with patient-derived HLA alleles identified 204 potential unique pMHCs for patient 2 (Supplementary Table 3). We therefore investigated the ability of patient 2 MHC class I–matched antigen-presenting cells (APCs) expressing brachyury to activate T cells harbouring the
identified TCR sequences (TCR1 comprised of TRA-1 and TRB; TCR2 comprised of TRA-2 and TRB) (Fig. 4a, b). To facilitate experiments, patient 2 MHC class I molecules were split into two sets (HLA Set1 and Set2), and these sets were delivered to synthetic APCs along with three TBXT isoforms (see 'Methods'). ELISPOT analysis revealed responses of both TCR alpha–beta pairs (TCR1 and TCR2) to synthetic APCs expressing either set of patient-derived HLA alleles and TBXT, with the strongest response associated with TCR1 in coculture with APCs expressing HLA Set2 and TBXT-202 (Fig. 4c). Investigation of individual HLA alleles revealed the greatest response of TCR1 T cells in the presence of HLA-C*04:01 (Fig. 4d). To identify specific brachyury epitopes that mediated T cell stimulation in our model system, we screened peptides that were predicted to bind HLA-C*04:01 (Supplementary Table 3).
We identified a peptide, RFKELTNEM, that induced T cell stimulation as measured by CD137 and interferon γ (IFNγ) expression, as well as elicited T cell-mediated target cell killing (Fig. 4e–g and Supplementary Fig. 3d, e). Together, our data indicate patient 2’s PDC tumour sample harboured a brachyury-specific CD8+ T cell clone. The presence of cytotoxic T cells and evidence for brachyury tumour antigen-mediated immune activity, along with high expression of PD-L1, were compatible with the notion that the observed responses to nivolumab may have been driven by disinhibition of this brachyury-specific T cell clone and potentially other tumour-reactive T cells.

Methylation profiles reveal common immune pathway activities between chordoma and a subset of RTs

Preliminary reports from phase I/II clinical trials in solid paediatric tumours have indicated a generally low rate of response to ICIs7–9. However, ICI responses documented in individual patients participating in clinical trials, or receiving off-label therapy, has pointed to a potentially sensitive group of SWI/SNF-mutant paediatric tumours9,10,13,34. For example, increased sensitivities to T-cell mediated killing and improved survival after ICI treatments observed in clear cell renal cell carcinomas have been linked to SWI/SNF mutations15,20. In particular, applications of immunotherapies such as CAR-T cell and ICI treatments in paediatric and adult INI1-deficient RTs have been described9,10,13,34–36. RTs are characterized by heterogeneous immune phenotypes, with a subset of tumours exhibiting immunologically hot phenotypes14,37. Given the common loss of INI1 in RT and PDC, and prevalence of CD8+ T cell infiltration in a subset of RTs and the two chordomas presented here, we sought to determine if paediatric chordomas were more similar to immunologically hot RTs than to immunologically cold RTs. DNA methylation clustering of chordoma and extra-cranial malignant RT (MRT) and cranial atypical teratoid RT (ATRT) cases using non-negative matrix factorization (NMF; Fig. 5a, b) and uniform manifold approximation and projection (UMAP) algorithms (Fig. 5c) consistently showed that chordoma cases clustered most closely with RT subgroup 4 characterized by higher infiltration levels of cytotoxic CD8+ T cells compared to the immunologically cold ATRT-SHH and -TYR subgroups. While hierarchical clustering of conventional chordoma and PDCs was consistent with previous observations that these are distinct molecular subgroups9 (Supplementary Fig. 4), both chordoma subgroups were similarly correlated with RT subgroup 4. RT subgroup 4 was largely comprised of extra-renal MRTs and characterized by gene expression and DNA methylation profiles that suggested increased immune response pathway activities and tumour-infiltrating T cells compared to other RT subgroups17. To explore molecular distinctions between chordomas and RT subgroups, we compared methylation profiles in chordomas to RT subgroups 1–3 and 5 (i.e. excluding subgroup 4). We observed enrichment of genes within differentially methylated regions that were involved in type I interferon response and response to viral infection pathways when chordomas were compared to RT subgroups 1–3 and 5 (Fig. 5d and Supplementary Table 4). These results are compatible with the notion that epigenetic dysregulation of immune pathways and increased CD8+ T cell infiltration may be a feature shared between chordoma and a subset of RTs.

DISCUSSION

Diverse immune profiles across paediatric solid tumour patients have been described37, and there is speculation that immunologically hot tumours may be particularly sensitive to ICI therapy as has been proposed for adult solid tumours32,38–41. Interim results from prospective trials have indicated limited responses to ICIs in paediatric solid tumours, and argued that PD-L1 expression on its own is likely insufficient to predict response across paediatric cancers7,9. Some paediatric patients with INI1-negative tumours have demonstrated clinical benefit from ICIs, including paediatric chordoma and RTs9,10,13. The beneficial response to the ICI, nivolumab, in a paediatric chordoma we describe here support these initial observations, and add further support for antigen-directed T cell responses and cytotoxic T cell infiltration that contribute to establishing an inflamed tumour microenvironment. Thus, our observations provide evidence that an immunologically hot phenotype is a compelling marker correlated with ICI response, and may provide mechanistic insight into the mediators of this response. Epigenomic analyses points to common immune related pathway activities in immunologically hot RTs and chordomas, indicating a shared immune phenotype that could potentially be exploited with immune targeting therapies as demonstrated for patient 2. The collective evidence from past studies and our observations presented here indicate that profiling of immune phenotypes as part of prospective clinical trials will be important to evaluate whether immune infiltrates are
Fig. 4  Patient-derived TCR activation and cell killing in response to a brachyury peptide antigen. a Unique TCR alpha (TRA) and TCR beta (TRB) chain sequences were inferred using bulk tumour RNA sequencing data from patient 2. The relative fraction of each sequence compared to all TRA and TRB aligned sequences is plotted on the y-axis. The two dominant TRA sequences TRA-1 and TRA-2 (red and orange bars, respectively) and the dominant TRB sequence (blue bar) were used for subsequent in vitro T cell activation ELISpot assays. b Schematic of ELISpot analysis of two reconstituted T cells (TCR1 and TCR2) expressing a patient-derived TRB chain and either of the two TRA chains, and synthetic antigen-presenting cells (APCs) transduced with one set of the patient’s classical HLA class I alleles (Set1 and Set2) and TBXT. IFNγ production is measured by the number of spot-forming units (SFU) per million cells. ELISpot results are shown for c TCR1 and TCR2 co-cultured with HLA Set1 or Set2 APCs expressing TBXT-201, 202, 203 or control (n = 3 for each condition, one way ANOVA; p values: TCR1 p = 2e−05 and TCR2 p = 0.00032). d TCR1 or control T cells and either HLA Set2 or HLA-A*31:01, HLA-B*44:03 or HLA-C*04:01 alleles (n = 3 for all conditions, unequal variance, two-sided T test Bonferroni corrected; p values for HLA Set2 p = 0.1, HLA-A*31:01 p = 0.0017, HLA-B*44:03 p = 1 and HLA-C*04:01 p = 0.014); e varying concentration of brachyury peptide RFKELTNEM with HLA-C*04:01 APCs with TCR1 T cells. Percentage of f CD137-positive T cells and g target cell death following co-culture with no peptide, HLA class I control CEF peptide pool or the RFKELTNEM in f, g). Box plots in c, d, f, g represent median, upper and lower quartiles, and whiskers represent limits of the distributions (1.5-times interquartile range).
predictive of ICI response in chordoma, RTs and paediatric malignancies more broadly.

Tumour antigens are potentially targetable mediators of anti-tumour immune responses, particularly in tumours with low TMB. A previous HLA-A*02:01 restricted brachyury epitope (WLLPGTSTL) has been shown to stimulate T cell-mediated cell killing, and brachyury-targeting T cells have been reported in chordoma patients including adult patients who have responded to immunotherapy. Given the absence of a single clonal nonsynonymous mutation in patient 2, aberrant expression of the brachyury tumour antigen was a strong candidate target for the anti-tumour response following treatment with nivolumab in patient 2. Our discovery of brachyury-targeting tumour-infiltrating lymphocytes in chordomas, along with expression of PD-L1 supports further investigation of similar patients with immune modulating therapies.

METHODS
Ethics and consent
This work was approved and conducted under the University of British Columbia Children’s and Women’s Research Ethics Board (H13–01640). All
patients presented in this publication were approached for enrolment by a
study trained oncologist and provided written informed consent.

Diagnostic biopsy preparation and staining/IHC

Needle core biopsies of tumour were fixed in 10% neutral buffered
formalin for a minimum of 8 h prior to automated tissue processing and
paraffin embedding. Four micron formalin-fixed paraffin embedded tissue
sections were cut by microtome and mounted onto charged glass slides.
Tissue sections were either subjected to automated routine staining with
haematoxylin and eosin, or subjected to IHC using the Ventana Benchmark
XT automated stainer. Antibody staining conditions were as follows: anti-
pan-keratin (clone AE1/AE3, MS-343-R7 Thermo Scientific, ready to use)
incubation at 37 °C for 32 min, anti-BAF47/INI1 (clone 25, 612110 BD
Bioscience, 1:100) incubation at 37 °C 32 min and anti-S100 (clone 4C4.9,
790–2914 Ventana, prediluted) incubation at room temperature 4 min.
For brachytherapy IHC (EPR18113, Abcam, 1:2000), staining was performed at
PhenoPath Laboratories (Seattle, WA).

PCR-free DNA library preparation

Nuclease acid was extracted from fresh frozen using RLT buffer (Qiagen).
Constitutional DNA representing normal cells was extracted from peripheral
blood. To minimize library bias and coverage gaps associated with PCR
amplification of high GC or AT-rich regions, a version of the
TruSeq DNA PCR-free kit (E6875–68778–GSC, New England Biolabs),
amplified on a Microlab NIMBUS liquid handling robot (Hamilton) was
employed. Briefly, 500 ng of genomic DNA was arrayed into wells in a
96-well microtitre plate and subjected to shearing by sonication (Covaris
LE220). Sheared DNA was end-repaired and size-selected using para-
magnetic PCRClean DX beads (C1003–450, Aline Biosciences), targeting a
350–450 bp size range. After 3’ A-tailing, full length TruSeq adapters were
ligated to DNA fragments. Libraries were purified using paramagnetic
beads (Aline Biosciences). Prior to sequencing, PCR-free genome library
concentrations were quantified using a qPCR Library Quantification kit
(KAPA, KK4824).

Bisulfite library construction

To track the efficiency of bisulfite conversion, 10 ng lambda DNA (Promega)
was spiked into 1 µg genomic DNA quantified using Qubit fluorimetry and
arrayed into a 96-well microtitre plate. DNA was sheared to a target size of
300 bp using Covaris sonication and the fragments were subjected to end-
repair and phosphorylation in a single reaction using an enzyme mixture
(New England Biolabs) containing T4 DNA polymerase, Klenow DNA
Polymerase and T4 polynucleotide kinase. End-repaired cDNA was
amplified using a Quant-iT dsDNA High Sensitivity Assay Kit on a Qubit fluorimeter (Invitrogen).

Strand-specific RNA-seq library preparation

Qualities of total RNA samples were determined using an Agilent
Bioanalyzer RNA Nanochip or Caliper RNA assay and arrayed into a 96-
well plate (Thermo Fisher Scientific). Polyadenylated (poly(A)) RNA
was purified using the NEBNext Poly(A) messenger RNA (mRNA) Magnetic
Isolation Module (E7490L, NEB) from 350 ng (patient 1) and 192 ng (patient
2) total RNA normalized in 35 µL for DNAs (1 treatment; Unit, Invitrogen).
DNase-treated RNA was purified using RNA MagCleat DX beads (Aline Biosciences,
1000-250) on a Microlab NIMBUS liquid handling robot (Hamilton
Robotics, USA). mRNA selection was performed using NEBNext Oligo(dT)25
beads (NEB). First-strand cDNA was synthesized from the purified
polyadenylated mRNA or rRNA depleted total RNA using the Maxima H
Minus First Strand cDNA Synthesis kit (Thermo-Fisher, FSP99760210) and
random hexamer primers at a concentration of 5 µM along with a final
concentration of 1 µg/µL Actinomycin D, followed by PCRClean DX bead
purification on a Microlab NIMBUS robot (Hamilton Robotics, USA). Second
strand cDNA was synthesized following the NEBNext Ultra Directional
Second Strand cDNA Synthesis protocol (NEB) which incorporates dUTP in
the dNTP mix, allowing the second strand to be digested using USERTM
enzyme (NEB) in the post-adapter ligation reaction, thus achieving strand
specificity. cDNA was fragmented using Covaris LE220 sonication to
achieve 200–250 bp average fragment lengths. The paired-end sequencing
library was prepared using a strand-specific, plate-based library construc-
tion protocol on a Microlab NIMBUS robot (Hamilton Robotics, USA). The
sheared cDNA was subject to end-repair and phosphorylation in a single
reaction using an enzyme premix (NEB) containing T4 DNA polymerase,
Klenow DNA polymerase and T4 polynucleotide kinase. End-replicated DNA
was purified in 96– well format using PCRClean DX beads, and 3’ A-tailed
(adenylation) using Klenow fragment (3’ to 5’ exonuclease). Illumina PE
adapters were ligated and adapter-ligated products were purified using
PCR Clean DX beads, then digested with USERTM enzyme (1 U/µL, NEB)
followed by 13 cycles of indexed PCR using Phusion DNA Polymerase
(Thermo Fisher Scientific Inc. USA) and Illumina’s PE primer set. The PCR
products were purified twice and size-selected using a 1:1 PCRClean DX
beads-to-sample ratio. The eluted DNA quality was assessed using the
Caliper LabChip GX for DNA samples and the High Sensitivity Assay
(PerkinElmer, Inc. USA). Quantification was performed using a Quant-it
DNA High Sensitivity Assay Kit on a Qubit fluorometer (Invitrogen).
Libraries were then pooled and size-selected to adjust the final library
molar concentration for sequencing.

Sequencing

Tumour genomes were sequenced on HiSeqX using v2.5 chemistry and
paired-end 150 base reads. Bisulfite-paired-end libraries were sequenced on
the HiSeqX to a length of 150 base reads. Transcripts were sequenced on
NextSeq500 using v2 chemistry to a length of 75 bp. Sequencing coverage data are listed in Supplementary Table 5.

DNA methylation analysis from WGBS

Bisulfite genomes were aligned to the human reference genome
(hg19) using novoalign (v.3.04.06) (http://www.novocraft.com/products/
novoalign/) and fractional methylation was assessed using in-house
novo5Mc (0.89d) (https://sv.bcgsc.ca/bitbucket/projects/EDCC/repo/
opencemt/browse/WGBS/QC/).

Gene expression

RNA-seq reads were aligned using STAR (v.2.5.2b) and expression
was quantified using RSEM (v.1.3.0) as TPM. Publicly available TCGA sramco
RNA-seq samples in Xena Public Data Hub (https://xena.ucsc.edu/public).
STAR and RSEM were generated from the hg38 reference genome (http://
 hgdownload.cse.ucsc.edu/goldenPath/hg38/bigZips/) and gene annotations
were based on Ensembl v.85. Samples used for gene expression analysis
are listed in Supplementary Table 1.

Somatic mutation detection

Sequence reads from normal and tumour whole genome libraries were aligned to the human reference genome (hg19) using the
Burrows–Wheeler Alignment tool (0.7.6a). Tumour genome sequences
were compared to those from the patient’s constitutional (normal) DNA to
identify somatic alterations. Regions of copy number variation and losses of heterozygosity were identified using the Hidden Markov model-based
approaches CNAseq2 (v0.0.6)50 and APOLOH (v0.1.1)51, respectively.
Somatic single nucleotide variants (SNVs) were identified using two approaches: (1) putative somatic variant calls from SAMtools52 (v0.1.17)
with subsequent scoring by machine-learning based MutationSeq53
(v4.3.5), and (2) identification and scoring with the joint caller Strelka54
(v1.0.6). Small (<20 bp) insertions and deletions (indels) were identi-
fied using assembly-based tools ABySS and Trans ABySS and alignment-based tools Manta55 v1.4.1056 and alignment-based tools ChimeraScan57 (v0.4.5) and DeFuse58
(v0.6.2); SVs in the DNA sequence data were identified using assembly-based tools
ABySS and Trans ABySS and alignment-based tools Manta55 v1.0.0 and

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Delly\textsuperscript{50} v0.7.3. Putative SV calls identified from the DNA and RNA sequences were merged into a consensus caller MAVIS\textsuperscript{61} (v2.1.1), where they were clustered, computationally validated and annotated against constitutional DNA to provide somatic and germline structural variant calls. Both DNA- and RNA-derived structural variant calls were additionally filtered to identify those called by more than one tool, and for which a contig could be assembled that aligned across a candidate genomic breakpoint. DNA SV calls were further filtered to exclude events with identical genomic breakpoints in multiple samples, removing potentially confounding germline variants and technical artefacts. Variants were annotated to genes using SNPEff (v3.2)\textsuperscript{93} with the Ensembl database (v69). TCR alpha and beta chains were detected from RNA-seq data using MixCR (v3.0.5)\textsuperscript{88}. POG570 solid tumour samples that were not obtained from lymph node, bone marrow or peripheral blood and had >1 unique TRB sequence detected were included in Supplementary Fig. 2b (n = 447).

**Immune cell deconvolution**

RNA-seq reads from paediatric chordomas (n = 2), the adult POG570 pancreatic cancer cohort (n = 570), TARGET RTs (n = 66), TARGET clear cell sarcoma of the kidney (n = 13), TARGET osteosarcoma (n = 87) and TCGA sarcoma data set (n = 255) were aligned and processed using JauguR (v2.0.3)\textsuperscript{85} as described in Jones and colleagues (2010)\textsuperscript{20}. Gene level RPKM (reads per kilobase of exonic sequence per million mapped reads) were calculated on the tumour WGS, tumour RNA-seq, and normal WGS data for each sample in order to generate a matrix that could be matched across different datasets. The median value across the matched samples was utilized for further analysis. The matrix was then filtered to exclude events with identical genomic breakpoints in multiple samples, removing potentially confounding germline variants and technical artefacts. Variants were annotated to genes using SNPEff (v3.2)\textsuperscript{93} with the Ensembl database (v69). TCR alpha and beta chains were detected from RNA-seq data using MixCR (v3.0.5)\textsuperscript{88}. POG570 solid tumour samples that were not obtained from lymph node, bone marrow or peripheral blood and had >1 unique TRB sequence detected were included in Supplementary Fig. 2b (n = 447).

**Immune cell deconvolution**

The TCR, HLA, and the TBXT sequences were sequentially cloned into pMND lentivirus vectors. For recombinant TCRs, the patient-derived TCR alpha and beta chains were synthesized de novo and cloned into lentiviral transfer plasmid as a bicistronic alpha-beta gene cassette containing a downstream mStrawberry reporter gene, giving two recombinant TCRs: TCR1 (TRA-1 CAVQSNYLKSLF; and TRB CSASTGRGTEAFF) and TCR2 (TRA-2 CALGAIVMDYAGLYTDKLFL; and TRB CSASTGRGTEAFF). The TBXT-201, TBXT-202 and TBXT-203 sequences were synthesized de novo and cloned into lentiviral transfer plasmids containing a downstream mStrawberry reporter gene. The HLA sequences were synthesized de novo and cloned into lentiviral transfer plasmids.

To generate each lentivirus, 80 μg of the specific transfer plasmid was incubated for 30 min at room temperature, with 72 μg of the pcMV-ΔR8.91plasmid, 8 μg of the pcMV-V5-G plasmid, and 430 μL of TransIT-LT2 transfection reagent made up to 500 μL with OptiMEM (Gibco, 31985062). HEK 293 T/17 cells (ATCC® CRL-11268\textsuperscript{53}) were transfected. Viral supernatants were collected and virus resuspended in OptiMEM. Titration and sorting of the transduced K562-APCs was done by staining the cells with HLA-A-PECY5 (Creative Diagnostic, DCAAH1-5290 150), HLA-B-APC (Creative Diagnostic, CABI-BL7610, 150) and HLA-C-PE (BD, 566372, 150) surface staining antibodies, along with the mStrawberry reporter transgene expressed with the recombinant TBXT transgenes. Representative images demonstrating the efficacy of lentiviral transduction and multiplicity of infection for TCR1, TBXT-201 and HLA-C*0401 constructs are shown in Supplementary Fig. 3a–c. Negative controls included untransduced K562 cells. Cells and antibodies were incubated at 4 °C for 30 min, then washed by adding 2 mL of FACS media (1× D-PBS 14190-144, Gibco with 2% HI-FBS 10082-147, Gibco) to each tube and centrifuging for 10 min at 400 × g at 4 °C, discarding the supernatant before resuspension in 400 μL of cold FACS media. The titration of TCR1 and TCR2 over K562 cells or the sorting of TCR1 and TCR2 transduced CD8\textsuperscript{+} T cells involved washing the cells after transduction by adding 2 mL of FACS media (1× D-PBS 14190-144, Gibco with 2% HI-FBS 10082-147, Gibco) to each tube and centrifuging for 10 min at 400 × g at 4 °C, discarding the supernatant before repeating the wash and resuspending the cells in 400 μL of cold FACS media. Acquisition of titration data was performed on the BD FACSsymphony cell analyser, and analysed using the FlowJo and GraphPad Prism software version 8.0.0 for windows (GraphPad Software, California, USA). For cell sorting, the BD FACSaria Fusion was used along with the FACSdiva software. FACs analysis involved capturing the data on FSC-A, FSC-H, FSC-W, SSC-A, SSC-H, SSC-W, 561 nm laser with the 670/14 filter (PE/CY5-HLA-A), the 640 nm laser with the 670/30 filter (HLA-B - APC), 560 nm laser with the 582/15 filter (PE-HLA-C), and 560 nm laser with the 640/20 filter (mStrawberry reporter transgene).

**Viral transduction and cell line generation**

Two separate K562 (ATCC, CCL-243) cell lines were developed expressing the patient’s six HLA class-I alleles: HLA-A-ABC Set1 and Set2 transduced with the TBXT lentivirus. Donor CD8\textsuperscript{+} T cells were isolated from a source of peripheral blood mononuclear cells (PBMCs) (Human Peripheral Blood Leukopak, Stemcell Technologies) using the Miltenyi MACS human T cell enrichment magnetic separation kit (Miltenyi Biotec, Auburn, CA) and analysed using the InForm and Vectra image analysis software (Perkin Elmer). Ten-to-12 images per slide taken from the same sample were analysed for each multiplex panel. Percentage of positive cells as determined by five independent algorithms were averaged for each image.

**Neoantigen prediction**

The TCR protein sequence was downloaded from Uniprot (U3KP65-1), Class I HLA genotypes for each patient were derived using OptiType\textsuperscript{96} run separately on the tumour WGS, tumour RNA-seq, and normal WGS data sets. NetMHCpan 4.0\textsuperscript{97} was used to predict pMHC binding for 8–11mer peptides from TBXT. NetMHCpan was run in Eluted Ligand mode, which predicts the likelihood that a peptide–MHC would be observed on the surface of a cell. We selected peptide–MHC combinations with Rank ≤2% as predicted binders.

**Design of TCR, HLA and TBXT sequences**

Recombinant TCRs were designed based on the RNA-seq data from patient 2 using Geneious (v8.1.4) and the IMGT/GENE Database\textsuperscript{86}. To minimize mismatch pairing with the endogenous TCRs, mouse constant regions were used for both the alpha and beta chains as previously described\textsuperscript{87}. Recombinant HLA s were designed based on the patient 2 derived HLA-class I alleles and the sequences were retrieved from the EMBL-EBI Immune Polymorphism Database\textsuperscript{84}. HLA-class I A, B and C alleles were split into two sets to facilitate testing (HLA Set-1: A*23:01, B*40:01, C*03:04; HLA Set-2 (A*31:01, B*44:03, C*04:01). The TBXT (ENSG00000164458) isofrom TBXT-201 and TBXT-202 CDNA reference sequences were retrieved from the NCBI Consensus Coding Sequence Database (CCDS ID: CCDS5290 and CCDS59045, respectively). The nucleotide sequence for TBXT-203 was retrieved from NCBI (Gene ID: NM_001366285.1).
ELISpots
ELISpot plates were coated with IFNγ capture antibody (2 µg/mL per well, Mabtech mAb 1-D1K, 3420-7) in D-PBS and then blocked with RPMI-1640 supplemented media. For experiments in Fig. 4c, d, APCs co-expressing HLA Set1, Set2 or individual alleles from Set 2 (HLA-A*31:01, HLA-B*44:03 or HLA-C*04:01) with 1 µg/mL of the RFKELTNEM peptide (GenScript). The peptide pulsed APCs were then cultured with TCR1 T cells at an E:T ratio of 1:1, followed by IFNγ detection as previously described. Figure 4b was created with BioRender.com.

CD137 activated expression and cytotoxic assays
For experiments in Fig. 4e, 5 × 10^4 (per reaction) APCs expressing the recombinant HLA-C*04:01 allele, were peptide pulsed with 10 µg/mL of the RFKELTNEM peptide (GenScript) or were left not peptide pulsed. For the CEF control, HLA Set2 APCs were peptide pulsed with 1 µg/mL of the CEF peptide. These APCs were then co-cultured with TCR1 T cells at an E:T ratio of 4:1 for 24 h before being stained with the CD8 AF-700 (clone SK1, 344724 BioLegend, 1:100) CD3-PE-Cy7 (clone 2C11, 48-0201-82, eBioscience, 1:200), CD137-APC (clone 4B4-1, A51019 ThermoFisher, 1:50) conjugated flow antibodies. Cells and antibodies were incubated at 4 ºC for 30 min, then washed by adding 2 mL of FACS media (1x D-PBS 14190-144, Gibco) with 2% HI-FBS 10082-147, Gibco) to each tube and centrifuging for 10 min at 400 x g at 4 ºC, discarding the supernatant before resuspension in 400 µL of cold FACS media. Acquisition was performed on the BD FACSymphony cell analyser, and analysed using FlowJo and GraphPad Prism software version 8.0.0 for windows (GraphPad Software, CA, USA). FACS analysis of the samples involved capturing the data on FSC-A, FSC-H, FSC-W, SSC-A, SSC-W, 637 nm laser with the 670/30 (APC-CD137 antibody) and 710/50 (AF-700-CD8) filters, 405 nm laser with the 450/50 (eFluor 450-CD3), and the 561 nm laser with the 610/20 (RFP) filter. A figure illustrating an example of this strategy is shown in Supplementary Fig. 3d.

For experiments in Fig. 4a, b, 5 × 10^4 (per reaction) APCs expressing the recombinant HLA-C*04:01 allele, were peptide pulsed with 10 µg/mL of the RFKELTNEM peptide (GenScript) or were left not peptide pulsed. For the CEF control, HLA set2 APCs were peptide pulsed with 1 µg/mL of the CEF peptide. These APCs were then co-cultured with TCR1 T cells at an E:T ratio of 4:1 for 24 h before being stained with the CD3-PE-Cy7 (clone SK1, 344724 BioLegend, 1:100) CD3-PE-Cy7 (clone 2C11, 48-0201-82, eBioscience, 1:200), CD137-APC (clone 4B4-1, A51019 ThermoFisher, 1:50) conjugated flow antibodies. Cells and antibodies were incubated at 4 ºC for 30 min, then washed by adding 2 mL of FACS media (1x D-PBS 14190-144, Gibco) with 2% HI-FBS 10082-147, Gibco) to each tube and centrifuging for 10 min at 400 x g at 4 ºC, discarding the supernatant before resuspension in 400 µL of cold FACS media. Acquisition was performed on the BD FACSymphony cell analyser, and analysed using FlowJo and GraphPad Prism software version 8.0.0 for windows (GraphPad Software, CA, USA). FACS analysis of the samples involved capturing the data on FSC-A, FSC-H, FSC-W, SSC-A, SSC-W, 637 nm laser with the 670/30 (APC-CD137 antibody) and 710/50 (AF-700-CD8) filters, 405 nm laser with the 450/50 (eFluor 450-CD3), and the 561 nm laser with the 610/20 (RFP) filter. A figure illustrating an example of this strategy is shown in Supplementary Fig. 3d.

DNA methylation analysis
IDAT files from Illumina 450k methylation arrays, published in Hasselblatt et al., were analysed using the minfi R package (v1.32.0). Raw data were normalized, and probes that exhibited a p value of >0.01, aligned to common SNPs, or were previously reported to be cross reactive were removed from the analyses.

Fractional methylation values from WGBS were filtered to include CpG sites that were commonly profiled by the Infinium HumanMethylation450 bead chip array. Unsupervised hierarchical clustering of paediatric chordoma fractional methylation was performed using the hclust and dist functions from the stats R package (v3.6.3) using 10,000 most variably methylated CpGs ranked by standard deviation. To visualize clustering results, a heatmap was generated using the heatmap3 R package (v1.1.7).

To reveal the extent of molecular similarities between RTs and SMARCBL1-deficient chordomas, we analysed WGBS data generated from the POG chordoma cases (n = 2), and DNA methylation array data from chordoma cases published in Hasselblatt et al. (2016; n = 23 cases), RT cases from Chun et al. (2019; n = 161 cases from cranial atypical teratoid RTs (ATRTs) and 131 cases from extra-cranial malignant RTs (MRTs)). We first selected CpG sites that were commonly profiled using WGBS, Illumina Infinium 450K HumanMethylation and 850K EPIC array platforms. Next, we analysed CpG sites that were considered to be positively methylated by first removing CpG sites with 0% methylation across all 317 samples, and then selecting CpG sites with beta-values greater than 0.3 in at least 10% of samples. These filtering steps yielded 248,121 CpG sites, which were then ranked using standard deviation to select the most variably methylated CpG sites. To reveal underlying clustering structures of the DNA methylation data, we performed an unsupervised NMF analysis using the top 10,000 most variably methylated CpG sites with a default Brunet algorithm, and 50 and 500 iterations for the rank survey and the clustering runs, respectively. The NMF method was implemented in the NMF R package (v0.20.2). The corresponding sample and subgroup information was visualized using the image function in R (v3.6.0). Unsupervised clustering using the UMAP algorithm was also performed on the 10,000 most variably methylated CpG sites using the umap R package (v0.2.6.0). The UMAP analysis was performed using 2,000 iterations during layout optimization (n_epochs), the minimum distance of 0.15 between points (min_dist) for better visualization of the global manifold structure of our data, and 20 nearest neighbours (n_neighbors) to allow 3–10% of the data set as the neighbours. For all other UMAP parameters, default settings were used.

Pathway enrichment
Fractional methylation of CpG sites that were common among paediatric chordoma samples from this study, and Hasselblatt et al. were compared to cranial and extra-cranial malignant RT methylation subgroups 1, 2, 3 and 5. Significantly differentially methylated CpGs were identified using Welch’s unequal variance T test. p Values were adjusted using the p.adjust R function with Bonferroni multiple hypotheses correction method in the stats R package (v3.6.3). Pathway enrichment analysis was performed using the methylgm function from the methylGSA R package using default settings (v1.4.9). The top 15 enriched gene sets as determined by adjusted p value were displayed.

Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this article.

DATA AVAILABILITY
Paediatric POG chordoma gene expression, DNA methylation and small mutation data for both paediatric chordoma patients sequenced at Canada’s Michael Smith Genome Centre can be downloaded from (https://www.bcgsc.ca/downloads/POG_chordoma/). Paediatric POG chordoma genomic and transcriptome sequencing data sets have been deposited at the European Genome-Phenome Archive (patient 1 EGAD000001008012 and patient 2 EGAD000001008013). Gene expression data for the POGS70 adult pan-cancer cohort can be downloaded from https://www.bcgsc.ca/downloads/POGS70/. POGS70 Genomic and transcriptomic data sets have been deposited at the European Genome-Phenome Archive (EGA, https://ega-archive.org/) as part of the study EGAS00000101159. Previously published gene expression data from The Cancer Genome Atlas (TCGA) used in Fig. 1 can be downloaded from https://xenabrowser.net/datapages/; Controlled TCGA data used in Fig. 2 was obtained from dbGaP (http://www.ncbi.nlm.nih.gov/gap). Previously published data by the Therapeutically Applicable Research to Generate Effective Treatments (TARGENT) initiative, phs000218, managed by the NCI were obtained from https://portal.gdc.cancer.gov/ and that by the Treehouse Childhood Cancer Initiative were obtained from University of California, Santa Cruz (https://treehousegenomics.soee.ucsc.edu/). All other
data supporting the findings of this study are available from the authors upon reasonable request.

**CODE AVAILABILITY**

The bioinformatics analyses were performed using open-source software, including Burrows–Wheeler alignment tool (v0.7.6a), CMAPseq (v0.0.6), APOLO (v0.1.1), SAMtools (v0.1.17), MutationSeq (v4.3.5), Strelka (v1.0.6), SNPeff (v3.2), ABYSS (v1.3.4), TransABySS (v1.4.10), Chimerascan (v0.4.5), Defuse (v0.6.2), Manta (v0.0.3), Delly (v0.7.3), MAVIS (v2.1.1), STAR (v2.5.2b), RSEM (v1.3.0), samtools (v0.1.17), CIBERSORT (v1.0.4), JAVAD (v2.0.3), MIXCR (v0.3.0) and VDtools (v1.1.9), OptiType (v1.3.1), NetMHCpan (v4.0), Geneious (v8.1.4), ADI ELOSP (v7.0), R (v3.6.0) and associated packages: minfi (v1.32.0), statis/v3.6.3, heatmap3 (v1.1.7), NMF (v0.20.2), umap(v0.2.6.0), methylGSAv1.4.9, novaAlign (v3.04.06), novomSC (v0.8.90). Additional processing involved in-house scripts that are available upon request.

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AUTHOR CONTRIBUTIONS

Article conception: L.M.W., R.J.D., S.R.R., E.T., M.H. Writing of main text: L.M.W. Manuscript editing: C.M.R., D.D.F., E.T., H.-J.E.C., S.B., E.P., M.H., M.K., R.A.H., B.H.N., S.R.R., R.J.D., M.A.M., S.J.M.J., D.R. Data collection and analysis: L.M.W., E.T., H.-J.E.C., S.B., E.P., M.H., P.D.J., M.K. In vitro T cell assay: C.M.R., D.D.F., R.A.H. Multiplex IHC: K.M., B.H.N. Figure construction: L.M.W., E.T., H.-J.E.C., K.M., D.G.R. Medical expertise and clinical history: S.R.R., R.J.D., D.G.R., D.D., M.H. Pathology: A.F.L., S.Y. Funding and support: J.L., M.A.M., S.J.M.J., B.H.N., R.A.H., M.H.

COMPETING INTERESTS

A patent application based on the work described in this study has been submitted to the United States Patent and Trademark Office (application number 63/200,483; authors L.M.W., C.M.R., D.D.F., E.T., R.A.H., S.J.M.J., J.L., S.R.R., R.J.D. and M.A.M. are listed as inventors). The remaining authors declare no competing interests.

ADDITIONAL INFORMATION

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Correspondence and requests for materials should be addressed to Marco A. Marra.

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