Genomic and immunologic characterization of INI1-deficient pediatric cancers

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Statement of Translational Relevance:

The prognosis for patients with INI1-deficient pediatric cancers is poor and therapeutic options, particularly in the relapsed or refractory setting, are limited. We sought to characterize the association between SMARCB1 genetic variants identified by next-generation sequencing and INI1 protein expression and to evaluate PD-L1 and CD8 expression in INI1-deficient tumors. Available evidence suggests a low rate of PD-L1 expression and response to immune checkpoint blockade in pediatric cancers. INI1-negative pediatric cancers may represent an important exception to this despite remarkably low tumor mutational burden. Our findings provide evidence supporting a novel strategy for treating INI1-deficient cancers. This study contributes to emerging data that immune checkpoint blockade either as monotherapy or in combination with other agents may be an effective treatment approach for these aggressive cancers.
Abstract

Purpose: Several aggressive pediatric cancers harbor alterations in SMARCB1, including rhabdoid tumors, epithelioid sarcoma and chordoma. As tumor profiling has become more routine in clinical care, we investigated the relationship between SMARCB1 genetic variants identified by next-generation sequencing (NGS) and INI1 protein expression. Therapeutic approaches for INI1-deficient tumors are limited. Early reports suggest a potential role for immune checkpoint inhibition in these patients. Thus, we also investigated PD-L1 and CD8 expression in INI1-negative pediatric brain and solid tumors.

Experimental Design: We performed immunohistochemistry (IHC) for INI1 and immune markers (PD-L1, CD8, and CD163) and NGS on tumor samples from 43 pediatric patients who had tumors with INI1 loss on previous IHC or SMARCB1 genomic alterations on prior somatic sequencing.

Results: SMARCB1 two-copy deletions and inactivating mutations on NGS were associated with loss of INI1 protein expression. Single-copy deletion of SMARCB1 was not predictive of INI1 loss in tumor histologies not known to be INI1-deficient. In the 27 cases with INI1 loss and successful tumor sequencing, 24 (89%) had a SMARCB1 alteration detected. Additionally, 47% (14/30) of the patients with INI1-negative tumors had a tumor specimen that was PD-L1 positive and 60% (18/30) had positive or rare CD8 staining. We report on 3 patients with INI1-negative tumors with evidence of disease control on immune checkpoint inhibitors.

Conclusions: A significant proportion of the INI1-negative tumors express PD-L1, and PD-L1 positivity was associated with extracranial tumor site. These results suggest that clinical trials of immune checkpoint inhibitors are warranted in INI1-negative pediatric cancers.
Introduction

Multiple aggressive pediatric cancers are characterized by alterations in SMARCB1, which is a core subunit of the SWItch/Sucrose Non-Fermentable (SWI/SNF) chromatin remodeling complex (1). Inactivation of the SMARCB1 gene and subsequent loss of INI1 protein expression is well described in several tumor types including rhabdoid tumors, epithelioid sarcoma, and chordoma (2-8). These rare tumor diagnoses are associated with a dismal prognosis, particularly in the relapsed or refractory setting, and few novel treatment options are available (9-14). Aside from SMARCB1 alterations, next-generation sequencing (NGS) of INI1-deficient tumors has not shown other recurrent genetic abnormalities (15,16).

Malignant rhabdoid tumors can arise in the brain, kidney, liver, and other soft tissues. These tumors, most often diagnosed in infants and young children, are highly resistant to treatment and are often fatal, with historical 5-year overall survival between 17% and 33% despite intensive therapy (10,12). Epithelioid sarcoma is a rare tumor that typically occurs in the extremities of adolescents and young adults. In the setting of relapse or recurrence there is no standard of care and prognosis is poor (6,17,18). Poorly differentiated chordomas with loss of INI1 expression are rare pediatric tumors which have been reported more recently and are also associated with a dismal prognosis (4,19).

EZH2 is essential for all cancers with alterations of subunits of the SWI/SNF complex (20). EZH2 targeted inhibitors are currently in clinical trials that are available to children and adults [NCT02601950, NCT02601937]. Genetic variants in SWI/SNF subunits and the results of IHC protein expression are among the clinical trial enrollment criteria. Partial responses to EZH2 inhibitors have been observed in patients with INI1-negative tumors, and thus, in specific diagnoses, loss of INI1 has been identified as a potential biomarker of response to this class of targeted therapy (21). As more pediatric cancers are subjected to tumor sequencing, genomic alterations in the SWI/SNF subunits will be identified. The association between these genetic variants and the IHC findings is not known. Conversely, the ability to detect the genomic alterations responsible for loss of INI1 expression with targeted NGS panels in common use has not been evaluated previously.

In the present study, we analyzed the relationship between SMARCB1 genetic variants identified on a NGS panel (OncoPanel) and INI1 protein expression (22). Beyond EZH2
inhibitors, therapeutic approaches for INI1-deficient tumors are limited. Thus, we also investigated the potential role for other targeted therapies by evaluating for other genomic events and for PD-L1 protein expression in a cohort of INI1-deficient pediatric brain and solid malignancies.

Materials and Methods

Patients and samples

This study was approved by the Dana-Farber Cancer Institute (DFCI) Institutional Review Board with a waiver of informed consent and was conducted in accordance with the U.S. Common Rule. Patients were identified for inclusion in the study by investigating two institutional databases in parallel. The Boston Children’s Hospital Pathology database was used to identify patients with tumor specimens collected between the years of 2000 and 2017 that had negative nuclear INI1 staining by immunohistochemistry (IHC). A database of somatic sequencing results from 280 pediatric patients with solid malignancies from the PROFILE Cancer Research Study was used to identify patients with SMARCB1 genomic alterations (single nucleotide variants, 1-copy deletions or 2-copy deletions) on panel sequencing (23).

Patients were included in the study if sufficient archival formalin-fixed paraffin-embedded (FFPE) tissue was available for both OncoPanel sequencing and IHC. A total of 43 patients were included in the study. One tumor block from each of the 43 patients was cut to obtain material for IHC for INI1, PD-L1, CD8 and CD163 and next-generation sequencing. A second tumor specimen was available for 10 of the 43 patients from a different time point during the patient’s disease course. These additional specimens were cut to obtain material for PD-L1 IHC.

Medical record review of the patients in the cohort was conducted to obtain clinical characteristics including diagnosis, sample timing, and lesion treatment. Sample timing was defined as either at initial diagnosis (which included local control surgery during upfront treatment) or at relapse/progression. Lesion treatment was defined as pre-treatment if the procedure to obtain the sample was performed at initial diagnosis prior to initiation of treatment or at relapse/recurrence before treatment of that specific relapse/recurrence. Lesion treatment was defined as post-treatment if the procedure was obtained during treatment with chemotherapy
or radiation (including local control surgery). The three patients treated with immune checkpoint blockade were identified through medical care provided in the pediatric oncology clinics of study authors (KAJ, EM and PL) following institutional guidelines for case reports. The clinical cases were selected from the case records of the Dana-Farber Cancer Institute and University of Texas Southwestern based on observed clinical response to immune checkpoint inhibitor and so these clinical cases are not entirely overlapping with the study cohort. Only one of these patients (P56) was included in the study cohort. The other two patients had IHC and NGS results available from clinical testing.

**IHC analysis**

Immunohistochemical stains for INI-1, PD-L1, CD8, and CD163 were performed on all cases using the Leica Bond according to standardized procedures on automated platforms. Antibody PD-L1, clone 9A11 was run at 1:100, using the Leica Biosystems Refine Detection Kit with citrate antigen retrieval (24), antibody CD163: clone MRQ-26, and antibody CD8: clone 4B11. Stains were interpreted by two pathologists (AA, JP) who were blinded to the sequencing results. INI1 stain was considered “retained” when all tumor cells had nuclear expression, while it was considered to be “totally lost/negative” when all nuclei lacked nuclear positivity and “partially lost” when some cells had retained nuclear expression. The extent of staining for PD-L1 was recorded as percentage in both tumor cells and tumor infiltrating lymphocytes (TILs) and was considered “positive” if there was ≥1% PD-L1 staining of the tumor cells or TILs. PD-L1 was considered negative if there was no staining. The cut-off point used for PD-L1 of ≥1% tumor cells or TILs was used for many clinical trials and is an FDA indication for immune checkpoint inhibitors in multiple diagnoses (25,26). Staining for CD8 and CD163 was assessed within the tumor and in the peri-tumor tissue. The extent of staining for CD8 and CD163 was recorded as “absent/negative” if there was no staining, “rare” if 1-20 cells per 20x field stained positive and “positive” if greater than 20 cells per 20x field stained positive.

**Next-generation sequencing**

Molecular profiling of tumor DNA was achieved via massively parallel sequencing (OncoPanel), as previously described (22,27). Briefly, DNA was extracted from FFPE specimens using standard extraction methods (Qiagen, Valencia, California) and quantified using
PicoGreen dsDNA detection (Life Technologies, Carlsbad, California). Targeted next-generation sequencing assessed 447 genes, using the TruSeq LT library preparation kit (Illumina, San Diego, California), a custom RNA bait set (Agilent SureSelect), and the Illumina HiSeq2500. Read analysis was conducted using Picard tools, the Genome Analysis Toolkit, Riker, MuTect and Oncotator. Sequence variants were filtered to exclude those that occur at a population frequency of greater than 0.1% in the gnomAD genome database (Broad Institute). Copy number variants were identified using RobustCNV, an algorithm in development at the Center for Cancer Genome Diagnostics at the Dana-Farber Cancer Institute. RobustCNV relies on localized changes in the mapping depth of sequenced reads including normalization algorithms. Structural variants were detected using the custom Breakmer algorithm, as previously described (28). Mutational burden, microsatellite stability, and mutational signatures were calculated, as previously described (23). Each alteration was reviewed manually by a molecular genetic pathologist. Clinical interpretation by a molecular pathologist includes tiered reporting of identified genetic alterations. As previously described, Tier 1 alterations are those with well-established published evidence of clinical utility in the tumor type sequenced. Tier 2 alterations are those that may have clinical utility. Tier 3 alterations are those with possible but uncertain utility and tier 4 alterations are those with unknown significance (23).

Gene Expression Analysis

Correlation of SMARCB1 and CD8A or PD-L1 mRNA levels was performed using Spearman correlation of mRNA levels from publicly available RNA-sequencing data across The Cancer Genome Atlas (TCGA) cancer types calculated by TIMER (Tumor Immune Estimation Resource) and adjusted for tumor purity (29). Correlations were considered significant at a p-value < 0.01.

Statistical analysis

Fisher exact test and unpaired t-test was used to compare clinical variables according to categories defined by PD-L1 status. P-values were considered significant at a p-value < 0.05. Data was analyzed using GraphPad Prism Version 8 (San Diego, CA, USA).
Results

Determination of study population

From the pathology database, 85 patients with INI1-negative tumors were identified. Of the 85 patients, 29 had tumor tissue available from previous clinical procedures for further studies. From the sequencing database, 23 patients with SMARCB1 alterations were identified, 17 of whom had tumor specimen available. The SMARCB1 alterations included single-copy deletion (n=14), two-copy deletion (n=2), and single nonsense mutation (n=1). Three patients with available tumor tissue were present in both databases. Thus, a total of 43 patients (26 from the pathology database, 14 from the sequencing database, and 3 from both) were included in the study cohort.

Clinical characteristics

The clinical characteristics of the 43 patients in the study cohort are summarized in Table 1. Within the pathology cohort, 6 tumor types were represented, of which the most common histologies were extrarenal rhabdoid tumor, atypical teratoid/rhabdoid (ATRT) tumor and malignant rhabdoid tumor of the kidney, comprising 79% of the cohort. One patient with each of those diagnoses was identified in both databases. In the sequencing cohort, a broader range of diagnoses were identified, with 1-2 patients per diagnosis. The majority of samples in the pathology cohort were obtained at initial diagnosis (69%) and prior to treatment (65%). In the sequencing cohort, 53% of samples were obtained at initial diagnosis and 76% of samples were obtained prior to treatment.

Correlation of SMARCB1 alterations and INI1 protein expression

Upon prospective IHC staining for INI1, 69% (n=30/43) of patients were found to have INI1-negative tumors (26 from the pathology cohort, 1 from the sequencing cohort, and 3 from both cohorts). Of the patients in the pathology cohort, all patients were confirmed to be INI1-negative. Panel NGS was successful in 91% (n=39/43) of the patients in the study cohort. In the sequencing cohort (n=17), two-copy deletion of SMARCB1 (n=2) as well as single nonsense mutations (n=1) were predictive of INI1 loss on IHC (Figure 1). In contrast, single-copy deletion of SMARCB1 on NGS was not predictive of loss of INI1 expression with only 1/10 (10%) of
SMARCB1 single-copy deletion cases from the sequencing cohort having loss of INI1 on IHC (Figure 1). In the 27 cases with INI1 loss by IHC and successful tumor sequencing, 24 (89%) had a genomic alteration in SMARCB1 on NGS and 3 (11%) had no SMARCB1 alteration detected. Of the 24 cases with a SMARCB1 alteration on sequencing, twelve cases (50%) had two-copy deletion, 5 (21%) had single-copy deletion, 5 (21%) had two inactivating alterations, and 2 (8%) had single nonsense mutations (Figure 2).

Additional genomic alterations in INI1-negative tumors

Very few additional genomic events were identified in the INI1-negative tumors, and all the Tier 1-3 alterations excluding low copy number amplifications are displayed in Supplementary Figure 1. The most common alterations in genes other than SMARCB1 were single-copy deletions in MAPK1, CHEK2, NF2, EWSR1, ZNRF3, EP2300, and CRKL. These genes are adjacent to SMARCB1 and upon review there was a combination of arm level and segmental deletions in the area of SMARCB1. Other genes containing variants tiered 1-3 in more than one of the confirmed INI1-negative cases were ARID1B, APC and TP53. On further review, the ARID1B variants are of uncertain significance being likely benign with respect to impact on protein function. On the other hand, the APC variants while also of uncertain significance are more likely pathogenic. Of note, 1 of the APC variants with a population frequency of 3.6% occurs in 2 cases.

PD-L1 status in INI1-negative tumors

Of the 30 patients with INI1-negative tumors, 47% (n=14/30) had at least one tumor specimen that was PD-L1 positive with ≥ 1% PD-L1 staining of the tumor cells or TILs (Figure 2). In the PD-L1 positive samples, 12 had PD-L1 staining only in tumor cells, 2 had PD-L1 staining only in TILs, and 1 had PD-L1 staining in both tumor cells and TILs (Supplementary Table 1). Only one of the nine ATRT patients had a PD-L1 positive tumor specimen. There was an increased frequency of PD-L1 positive tumor samples in the extracranial tumors as compared to the intracranial tumors (p=0.017; Table 2). The median tumor mutational burden (TMB) was 4.1 mutations per megabase of DNA in the PD-L1 positive tumors, and 3.7 in the PD-L1 negative tumors. There was no association between PD-L1 status and timing of tumor sampling, prior treatment, or TMB (Table 2). Ten patients had specimens available at more than one time...
point relative to diagnosis/relapse and pre/post-treatment status for determination of PD-L1 status. There was no consistent pattern of PD-L1 status by sample time point (Supplementary Figure 2).

**Immune cell infiltration in INI1-negative tumors**

INI1-negative tumors were also assessed for immune cell infiltration via CD8 and CD163 IHC within the tumor and in the peri-tumoral tissue (Supplementary Table 1). Of the 30 patients with INI-negative tumors, 16% (5/30) had a tumor specimen with positive CD8 staining (greater than 20 cells per 20x field) and 43% (13/30) with rare CD8 staining (1-20 cells per 20x field) (Figure 2). Notably, all 5 of tumor specimens that were CD8 positive were in tumors that were also PD-L1 positive. CD163 staining was positive (greater than 20 cells per 20x field) in 83% (25/30) of the INI1-negative tumor specimens and was rare (1-20 cells per 20x field) in 10% (3/30). There was no association between CD163 staining and PD-L1 status.

**TCGA Analysis of SMARCB1 and Immune Microenvironment**

To evaluate the relationship between SMARCB1 gene expression and the immune microenvironment across human cancer, we examined the relationship between SMARCB1 and CD8A or CD274 (PD-L1) gene expression. Using TCGA RNA Sequencing and TIMER, we found an inverse correlation between mRNA levels for SMARCB1 and both CD8A and PD-L1 in human cancer types (Figure 3) (29). This is consistent with our finding of increased PD-L1 expression in INI1-deficient tumors.

**Patients Treated with Immune Checkpoint Inhibition**

An 18-year-old male (P56) presented with a forearm soft tissue mass and upon biopsy was diagnosed with epithelioid sarcoma with the tumor having loss of nuclear INI1 by IHC (Figure 4A). Initial staging work up revealed Stage III (T2b, N1, M0) diagnosis with PET avid clinically involved axillary lymph nodes and four of five lymph nodes involved by epithelioid sarcoma on axillary lymph node dissection. He was treated with neoadjuvant pazopanib and concurrent radiation therapy to the primary tumor and regional lymph nodes (48 Gray) followed by two large surgical excisions of the primary tumor both with microscopic positive margins. IHC staining for PD-L1 was negative on the tumor specimen from his initial diagnosis prior to
treatment and was positive (40% of tumor cells and 30% of peritumor lymphocytes) on the tumor specimen from his surgical resection following neo-adjuvant pazopanib and radiation (Figure 4B). Approximately 7 months after surgery, he experienced local disease recurrence and had multiple enlarging sub-centimeter cystic lung nodules concerning for distant metastatic disease. He was treated with an immune checkpoint inhibitor (pembrolizumab 200mg IV every 3 weeks) for 12 months (18 cycles) with stable disease. He then developed palpable lymph nodes and was started on intralolesional talimogene laherparepvec (T-VEC) injections into his right arm and axillary nodules every 3 weeks combined with continued systemic pembrolizumab. He remains on this treatment regimen after 8 months (10 cycles) with stable disease and has had a decrease in the measured size of the treated lesions with an 18.5% reduction in the largest 1.8cm tumor after 2 cycles, which was no longer palpable after 4 cycles (Figure 4C-D).

A 10-year-old female presented with neck pain and swelling and upon biopsy was diagnosed with anaplastic chordoma of the spine with nuclear loss of INI1 expression by IHC (Figure 4E). Further evaluation revealed metastatic pulmonary disease. Clinical next-generation sequencing (Foundation One) revealed SMARCB1 loss, with stable microsatellite status and low tumor mutational burden (4 Mut/mb). She was initially treated with an aggressive regimen of combination chemotherapy including ifosfamide, doxorubicin, cyclophosphamide, carboplatin and etoposide and local control with proton beam radiation. Approximately 6 months after completing therapy, she experienced metastatic relapse in the lung. She was treated on a clinical trial with an EZH2 inhibitor which was stopped due to disease progression with a new metastatic lesion in her thoracic spine. The thoracic lesion was treated with resection followed by proton beam radiation with good control locally but with progressive disease in her lungs. IHC staining for PD-L1 was positive (5% of tumor cells) on the relapsed tumor specimen (Figure 4F). She was then treated with an immune checkpoint inhibitor (nivolumab 3mg/kg IV every 2 weeks) with a partial response in some lesions (Figure 4G-H) and overall disease control for 9 months. She then received stereotactic body radiation therapy (SBRT) to two growing lung lesions and one cervical spine lesion combined with continued nivolumab with subsequent shrinkage of the radiated as well as multiple non-radiated lesions in the lung. Treatment with nivolumab was discontinued after a total of 14 months due to pneumonitis which responded to systemic steroids. She continued to have stable disease off of nivolumab for 12 months after the occurrence of pneumonitis, and then had progressive disease in the lung. She was re-challenged with
nivolumab which was stopped after 3 doses due to recurrent pneumonitis, which was again responsive to steroids. She has had stable disease off of nivolumab for 6 months after the second occurrence of pneumonitis.

A 5-month-old male presented with asymmetric soft tissue masses on his back and was diagnosed with malignant rhabdoid tumor with the tumor having INI1 loss on IHC (Figure 4I). Clinical next-generation sequencing (Foundation One) revealed SMARCB1 loss, with stable microsatellite status and low tumor mutational burden (2 Mut/mb). Staging work up revealed advanced metastatic disease with multiple soft tissue and bony sites of disease involvement. The family declined conventional cytotoxic chemotherapy and he was started on treatment with pembrolizumab (2mg/kg IV every 3 weeks). He was treated with pembrolizumab monotherapy for 15 weeks (5 cycles) with stable soft tissue sites of disease (Figure 4J-L). After 5 cycles he had progressive disease in his cervical spine for which he was treated with radiation and continued pembrolizumab for 2 additional cycles. Treatment was stopped after Cycle 7 of pembrolizumab due to continued progression of the cervical lesion. He died from disease progression 6 weeks after his last dose of pembrolizumab at approximately 11 months of age.

Discussion

In summary, we have shown that SMARCB1 2-copy deletions and inactivating mutations are associated with loss of INI1 protein expression, however single-copy SMARCB1 deletion on NGS is not predictive of loss of protein expression. Panel NGS was able to identify alterations in SMARCB1 in the vast majority of tumors with INI1 loss by IHC. Alterations in SMARCB1 were identified in 89% (24/27) of those with successful sequencing.

We have further demonstrated that 47% (14/30) of patients with INI1-negative tumors of multiple histologies had positive PD-L1 staining despite low TMB. Interestingly, PD-L1 expression was associated with extracranial tumor site. Additionally, 60% (18/30) had positive or rare CD8 staining suggesting some degree of immune infiltration in the majority of INI1-negative tumors. Our results are in keeping with a recently published report of 16 cases of malignant rhabdoid tumor where 50% (8/16) showed membranous expression of PD-L1 in 10-70% of tumor cells (30). Previous studies have also shown immune infiltrates in ATRT and extracranial rhabdoid tumor samples as well as response to immune checkpoint blockade in a genetically engineered mouse model of rhabdoid tumor (31). Previous reports suggest a variable
rate of PD-L1 and CTLA-4 expression in pediatric cancers and a low rate of response to immune checkpoint blockade (32). The presence of PD-L1/PD-1 and CTLA-4 expression and response to immune checkpoint inhibitor therapy has, in lung cancer and melanoma, been correlated with high TMB (33,34). INI1-deficient cancers in children have, of all cancers, amongst the lowest mutational burden (30,35,36). Importantly, patients with PD-L1 expressing tumors of multiple histologies have improved responses and survival with immune checkpoint inhibition compared to patients with PD-L1 negative tumors (37).

The three cases reported in this study add to an expanding literature of patients of INI1-negative tumors benefiting from immune checkpoint inhibitor therapy. A pediatric patient with INI1-negative malignant rhabdoid tumor enrolled on a clinical trial of atezolizumab was reported to have a response (38,39). There is a published report of a 24-year-old young adult with metastatic INI1-negative epithelioid sarcoma progressing on pazopanib who had a partial response to nivolumab given with pazopanib (40). Another young adult patient with recurrent INI1-negative renal medullary carcinoma had a complete response to nivolumab lasting greater than nine months despite low TMB (41). Two subsequent adult patients with INI1-negative renal medullary carcinoma treated with nivolumab did not have durable responses (42). A single-arm phase II trial of nivolumab plus ipilimumab in adults with INI1-negative renal medullary carcinoma in adults is now open [NCT03274258] (43). PD-L1 expression and responses to immune checkpoint blockade have also been seen in cancers harboring loss of SMARCA4, another subunit of the SWI/SNF complex, despite low TMB (44,45).

Further studies are needed to elucidate the mechanism potentially linking loss of INI1 expression to PD-L1 expression and response to immune checkpoint inhibition. Previously, it was shown that inactivation of other members of the SWI/SNF complex (Pbrm1, ARID2, and BRD7) sensitized tumor cells to T cell killing most likely from role of the SWI/SNF complex in regulating chromatin accessibility of interferon-gamma inducible genes (46). Loss of SMARCB1 may lead to PD-L1 expression and sensitivity to immunotherapy from a similar mechanism.

Along with recently published reports, our study supports the development of clinical trials of immune checkpoint inhibitors in pediatric patients with INI1-negative malignancies which portend a poor prognosis and have limited treatments available. EZH2 inhibition has emerged as an attractive treatment to combine with immune checkpoint blockade based on the immunologic effects seen in both regulatory T cells and tumors, including increased PD-L1 expression, in the
setting of EZH2 inhibition (47-49). Furthermore, there was a recently published report of a patient with an aggressive INI1-negative chordoma who had a remarkable and durable response to sequential treatment with an EZH2 inhibitor, radiation, and immune checkpoint inhibition (47). Given the dependence of INI1-deficient tumors on EZH2, the combination of immune checkpoint inhibition and EZH2 inhibition may be a promising combination strategy for these difficult to treat cancers (50,51).
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References:


### Table 1. Clinical characteristics of the study cohort

<table>
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<tr>
<th>Clinical characteristics</th>
<th>Pathology Cohort (n=29)</th>
<th>Sequencing Cohort (n=17)</th>
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<td>Relapse/progression</td>
<td>9 (31)</td>
<td>8 (47)</td>
</tr>
<tr>
<td>Treatment timing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-treatment</td>
<td>19 (65.5)</td>
<td>13 (76)</td>
</tr>
<tr>
<td>Post-treatment</td>
<td>9 (31)</td>
<td>4 (24)</td>
</tr>
<tr>
<td>Unknown</td>
<td>1 (3.5)</td>
<td></td>
</tr>
</tbody>
</table>

*Three patients identified in both pathology and sequencing cohorts.*
Table 2. Characteristics associated with PD-L1 Status in INI1-Negative Tumors

<table>
<thead>
<tr>
<th></th>
<th>PD-L1 Positive (n=14)</th>
<th>PD-L1 Negative (n=16)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tumor Site, n (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extracranial tumors</td>
<td>13 (93)</td>
<td>8 (50)</td>
<td>0.017</td>
</tr>
<tr>
<td>Intracranial tumors</td>
<td>1 (7)</td>
<td>8 (50)</td>
<td></td>
</tr>
<tr>
<td><strong>Sample timing, n (%)</strong></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Initial diagnosis</td>
<td>10 (71)</td>
<td>12 (75)</td>
<td></td>
</tr>
<tr>
<td>Recurrence/progression</td>
<td>4 (29)</td>
<td>4 (25)</td>
<td></td>
</tr>
<tr>
<td><strong>Treatment, n (%)</strong></td>
<td></td>
<td></td>
<td>0.23</td>
</tr>
<tr>
<td>Pre-treatment</td>
<td>7 (50)</td>
<td>13 (81)</td>
<td></td>
</tr>
<tr>
<td>Post-treatment</td>
<td>6 (43)</td>
<td>3 (19)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>1 (7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>TMB</strong></td>
<td></td>
<td></td>
<td>0.61</td>
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<tr>
<td>Median</td>
<td>3.04</td>
<td>2.28</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>2.3-9.1</td>
<td>0.8-6.8</td>
<td></td>
</tr>
</tbody>
</table>
Figure Legends

**Figure 1:** Confirmatory *SMARCB1* Sequencing and INI1 Expression in Sequencing Cohort. A. Two cases with 2-copy gene deletion and one case with a nonsense mutation were identified. Repeat sequencing was successful and identified *SMARCB1* alterations for 2 of the 3 cases and INI1 IHC was negative in all 3 cases. B. Fourteen cases with 1-copy gene deletion were identified. Repeat sequencing was successful for 13 cases and *SMARCB1* alterations were confirmed in 10 cases. INI1 IHC was positive for 13 cases and negative for 1 case.

**Figure 2.** Clinical and Genomic Features of INI1-Negative Tumors. OncoPrint of *SMARCB1* alterations, tumor mutational burden (TMB), PD-L1 and CD8 staining and clinical features including diagnosis, sample timing and lesion treatment in 30 INI1-negative tumors.

**Figure 3.** Expression of *SMARCB1* is Inversely Correlated with *CD8A* and *PD-L1* mRNA levels in The Cancer Genome Atlas (TCGA) data. Volcano plot showing the Spearman’s correlation and estimated significance of *SMARCB1* with *CD8A* (left) and *PD-L1* (right) mRNA levels from RNA sequencing data across TCGA cancer types calculated by TIMER. Each dot indicates a cancer type in TCGA and red dots denote significant correlations (p < 0.05).

**Figure 4.** Patients Treated with Immune Checkpoint Inhibition. A-D Epithelioid sarcoma case. A. INI1 immunohistochemistry (IHC) from initial diagnosis showing negative nuclear INI1 in tumor cells at 40x (400 x magnification). B. PD-L1 IHC from resection after neoadjuvant pazopanib and radiation therapy showing PD-L1 staining in tumor cells and peritumor lymphocytes. C. Cutaneous lesions prior to talimogene laherparepvec (T-VEC) injections. D. Cutaneous lesions decreased in size after 3 cycles of T-VEC and continued pembrolizumab treatment. E-H Chordoma case. E. INI1 IHC from initial diagnosis showing negative INI1 staining in tumor cells. F. PD-L1 IHC from resection after relapse showing PD-L1 staining in 5% of tumor cells. G. Computerized Tomography (CT) scan showing pulmonary nodule (arrow) measuring 1.2cm before treatment with nivolumab. H. CT scan showing persistent response of the same pulmonary nodule after 28 cycles of treatment with nivolumab. I-L Malignant Rhabdoid tumor. I. IHC from initial diagnosis showing negative INI1 staining in tumor cells. J. Magnetic resonance imaging (MRI) of soft tissue lesion (arrow) measuring 14.3mm by 8.9mm at the time of diagnosis. K. MRI of same lesion (arrow) measuring 12.2mm by 8.0mm after 6 weeks of treatment with pembrolizumab. L. MRI of same lesion (arrow) measuring 9.4mm by 6.9mm after 14 weeks of pembrolizumab therapy.
Figure 1

A.  
- **SMARCB1 2 DEL (N=2)**
- **SMARCB1 Nonsense (N=1)**

  - Confirmed
    - No (N=1*)
      - INI1 Loss
        - No (N=0)
        - Yes (N=1)
    - Yes (N=2)
      - INI1 Loss
        - No (N=0)
        - Yes (N=2)

*One SMARCB1 2 DEL specimen with failed sequencing

B.  
- **SMARCB1 1 DEL (N=14)**

  - Confirmed
    - No (N=4*)
      - INI1 Loss
        - No (N=4)
        - Yes (N=0)
    - Yes (N=10)
      - INI1 Loss
        - No (N=9)
        - Yes (N=1)

*One SMARCB1 1 DEL specimen with failed sequencing
Figure 2
Figure 3

**SMARCB1 to CD8A**

- LGG
- PRAD
- LUSC
- LUAD
- SKCM
- OV
- BRCA
- THCA
- ESCA
- PAAD
- READ
- UCEC
- COAD
- CESC
- GBM

**SMARCB1 to PD-L1**

- LGG
- PRAD
- KIRC
- HNSC
- GBM
- CESC
- SKCM
- UCEC
- LIHC
- UCEC
- LUSC

*p value (log10)*

Spearman Correlation
Figure 4

A. 40x
B. 40x
C.
D.
E. 40x
F. 40x
G.
H.
I. 40x
J. 8.9mm, 14.3mm
K. 8.0mm, 12.2mm
L. 6.9mm, 9.4mm
Genomic and immunologic characterization of INI1-deficient pediatric cancers

Suzanne J. Forrest, Alyaa Al-Ibraheemi, Duong Doan, et al.

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