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SM Journal of Sarcoma Research

Research Article

RNA-Expression Profiling Reveals Immunotherapy Targets in Sarcoma

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Article Information

Received date: Nov 27, 2018
Accepted date: Dec 11, 2018
Published date: Dec 17, 2018

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Keywords Sarcoma; Undifferentiated pleomorphic sarcoma; Immunotherapy; CD276; CSF1R; PD-L1; PD-L2; TGFB1; OX40; Epithelial to mesenchymal transition; Tumor infiltrating macrophages; Inflamed; Non-inflamed

Abstract

Introduction: In sarcoma, no attention has been given to targets beyond the PD-1/PD-L1 axis. We profiled the immune microenvironment of sarcoma with a focus on Undifferentiated Pleomorphic Sarcoma (UPS), to identify novel immunotherapy targets.

Materials and Methods: Microsatellite instability, Tumor Mutation Burden (TMB), PD-L1 IHC and RNA-seq of 395 immune-focused transcripts were performed in 63 sarcomas, including 21 UPS. The expression of each transcript in the 63 sarcomas and a 1295 non-sarcoma solid tumor population was ranked against 167 cases of a diverse solid tumor reference population. The sarcoma (UPS and non-UPS) expression ranks were compared against the expression ranks of the 1295 non-sarcoma tumors using the Wilcoxon Rank-Sum test.

Results: Thirty eight percent and 19% UPS and non-UPS, respectively, were PD-L1 positive by IHC. Microsatellite instability and high TMB were not seen in any specimen. Compared to the 1295 non-sarcoma tumors, the immune therapy targets CD276 (B7-H3) and TGFB1 were significantly over expressed in UPS and non-UPS. The epithelial to mesenchymal transition-related SNAI2, TWIST, and ZEB1 were over expressed in both groups. UPS over expressed PD-L2, CSF1R, CD68 and CD163, while non-UPS over expressed OX40. "Inflamed" sarcomas tended to contain abundant CD8 transcripts and were more frequently PD-L1 IHC positive while "cold" sarcomas tended to be metastatic and non-UPS.

Discussion: A substantial subset of UPS and non-UPS are positive for PD-L1 IHC. The high expression of PD-L2 in UPS suggests that the PD-1/PD-L1 axis may be important in UPS. CD276 (B7-H3) and TGFB1 are other possible immunotherapy targets in sarcoma. CSF1R and OX40 are possible immunotherapy targets in UPS and non-UPS respectively. Myeloid suppression may be an immunosuppressive mechanism in UPS. Epithelial to mesenchymal transition is a possible mechanism of immune suppression in both UPS and non-UPS. Sarcomas can be categorized in "inflamed" and "cold" categories which associate with histological category, metastatic, and PD-L1 status.

Introduction

The probability of response to a checkpoint inhibitor is closely related to the nature of a tumor's immune microenvironment. Non-small cell lung cancer has emerged as the prototypical tumor type that is responsive to checkpoint inhibition and has a PD-L1 immunohistochemical (IHC) companion diagnostic biomarker which is used to determine whether pembrolizumab immunotherapy will be given [1,2]. In contrast, the sarcoma immune microenvironment is still poorly understood and biomarkers predictive of immunotherapy response are greatly needed.

Sarcoma diagnoses are rare and published case series typically lump heterogeneous sarcomas together. Due to this and because of the use of various different PD-L1 antibodies in different studies, the reported incidence of IHC PD-L1 positivity in sarcomas has varied greatly in the literature, ranging from 0% to 60% [3,4,13-15,5-12]. In soft tissue sarcomas, high PD-L1 expression by RNA-seq is associated with shorter metastasis-free survival [16]. In two meta-analyses the expression of PD-L1 was found to be a poor prognosticator in sarcomas [17,18].

Checkpoint inhibitors have yielded mixed results in sarcoma. Ipilimumab had no activity in 6 patients with synovial sarcoma [19]. When given at least 4 cycles of nivolumab, partial response or stable disease was seen in 12 of 24 patients with various sarcomas [20]. Ben-Ami reported that none of 12 uterine leiomyosarcoma patients responded to nivolumab [21]. Two patients with alveolar soft part sarcoma responded to durvalumab and durvalumab/tremelimumab. One of these neoplasms was PD-L1 positive while the other one was PD-L1 negative. Neither neoplasm had an elevated TMB [22]. Two of four patients with alveolar soft part sarcoma were reported to have partial response to anti-PD-L1 therapy [23]. A microsatellite stable, low TMB chondrosarcoma with 1% PD-L1 positivity was reported to respond to nivolumab [24]. Finally, the SARC028 clinical trial reported that 18% of patients with soft tissue sarcomas had an objective response to pembrolizumab. Twenty percent of patients with liposarcoma, 10% with synovial sarcoma, and 5% with bone sarcoma had a response. No response was seen in patients with leiomyosarcoma or Ewing sarcoma. Importantly,

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4 of 10 patients with UPS had a response and 2 of these 4 patients had at least 1% neoplastic cell PD-L1 positivity. Tumors from all 6 pembrolizumab unresponsive UPS patients were PD-L1 negative [9].

High RNA expression of B7-H3, TGFB1, and TIM3, which are putative immunotherapy targets, has been described in sarcoma, specifically in dedifferentiated liposarcoma, undifferentiated pleomorphic sarcoma, and myxofibrosarcoma [25].

In this study, we aimed to confirm previous findings and identify new targetable immune targets in sarcoma by RNA-sequencing expression profiling.

Materials and Methods

The specimens were evaluated for PD-L1 22C3 IHC (Agilent Technologies, Santa Clara, CA), microsatellite instability (RPCCC, Buffalo, NY), TMB (MiSeq, Illumina, San Diego, Ca) and expression of 395 immune-focused gene transcripts (Thermo Fisher Scientific, Waltham, MA). The RNA-seq component has been previously described [26]. A positive PD-L1 IHC result was defined as at least partial membranous staining in at least 1% of the neoplastic cells. Microsatellite instability was defined as instability in at least 2 of 5 microsatellites. High TMB was defined as at least 7.1 mutations per megabase.

A population of 167 solid tumor specimens was used to produce normalized rankings of the 395 gene transcripts. Details on this population have been published [26,27]. Using the Wilcoxon Rank-Sum test, the expression ranks of two sarcoma categories, UPS and non-UPS, were compared against the expression ranks of non-sarcoma solid tumors. Benjamini-Hochberg corrected p-values are reported (Figure 1). P-values less than 0.05 were considered significant.

Expression ranks were used to perform unsupervised clustering. The dissimilarity of hierarchical clustering of all transcripts was evaluated by Pearson correlation. Histology (UPS and non-UPS), primary/metastasis/recurrence status, CD8 expression, and PD-L1 IHC status annotations were added.

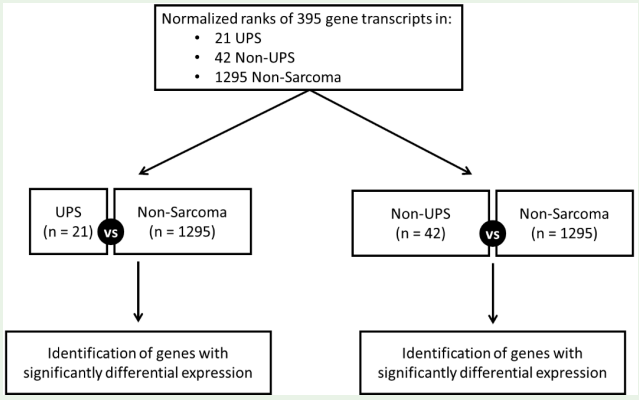

Figure 1: Flow chart of the analysis performed in this study. All transcripts from all samples (UPS, non-UPS, and non-sarcomas) were ranked against a separate population of 167 solid tumors (this population is not shown). The resultant ranks of UPS and non-UPS were compared against a large non-sarcoma solid tumor population to identify genes with differential expression in UPS and non-UPS.

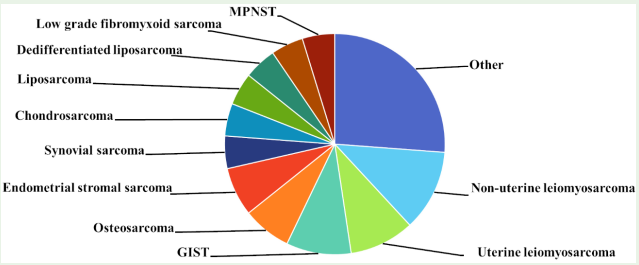

Figure 2: Breakdown of non-UPS included in this study.

Results

Sixty three sarcomas and 1295 formalin fixed paraffin embedded non-sarcomas were RNA-sequenced. The 1295 non-sarcoma specimens consisted of various tumor types including non-small cell lung carcinoma, colorectal carcinoma, endometrial carcinoma, urothelial carcinoma, pancreatic carcinoma, ovarian carcinoma, breast carcinoma, salivary gland carcinoma, thymic carcinoma, melanoma and others.

The 63 sarcomas included 21 UPS and 42 non-UPS paraffin embedded specimens from adult patients. The non-UPS set included various sarcoma types (Figure 2, Supplementary table 1). All cases had at least 50% neoplastic cell cellularity. The patient's age at the time of biopsy/resection ranged 24-84 years. The year of tissue procurement ranged from 2011 to 2018.

PD-L1 IHC results are shown in Figure 3 and Supplementary Table 1.

None of the 45 cases with complete microsatellite testing were microsatellite unstable. None of the 63 sarcoma cases had an elevated TMB (Supplementary Table 1).

CD276, TGFB1, SNAI2, TWIST, and ZEB1 were significantly over expressed in UPS and non-UPS compared to the non-sarcoma population. Compared to the non-sarcoma population, CSF1R, PD-L2, CD68, and CD163 were significantly over expressed in UPS. Similarly, OX40 was significantly over expressed in non-UPS (Figure 4, Supplementary Tables 2 and 3).

PD-L1 was significantly under expressed in non-UPS compared to the non-sarcoma population but there was no difference in expression

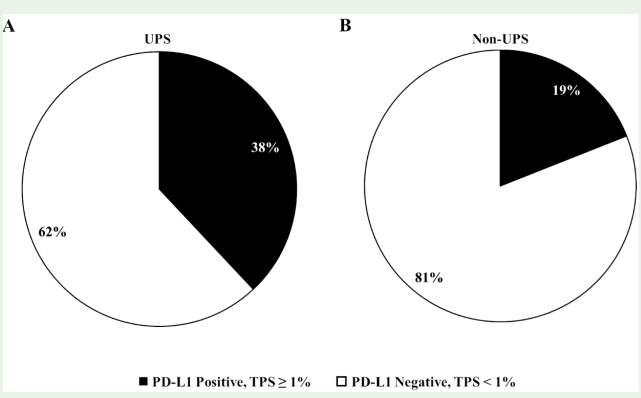

Figure 3: Breakdown if PD-L1 IHC status in UPS (A) and non-UPS (B).

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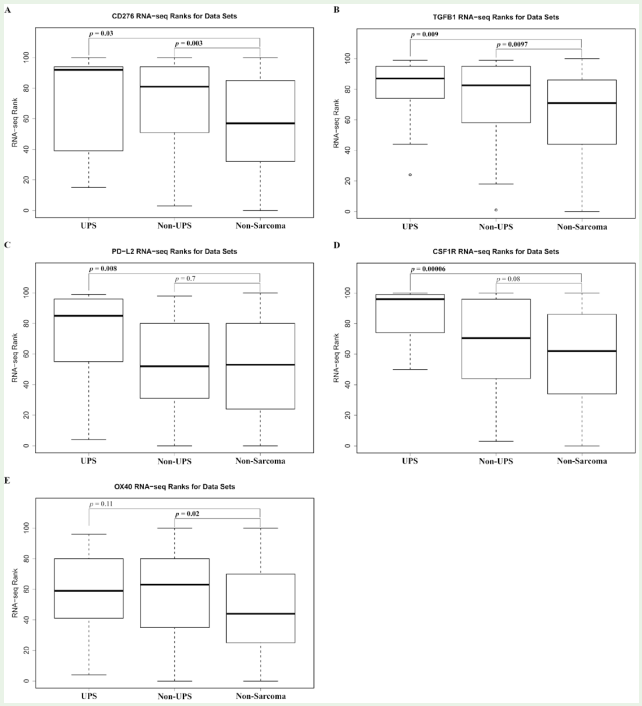

Figure 4: Box plots of CD276, TGFB1, PD-L2, CSF1R and OX40 in UPS, non-UPS, and non-sarcoma reference population. The top bars contain the adjusted p-values comparing the expression difference between these populations. Significant p-values (p<0.05) are bolded.

between the UPS and non-sarcoma population (Supplementary Tables 2 and 3).

Unsupervised clustering of all transcripts for all cases revealed three clusters: cluster 1 with the least transcript over expression and cluster 3 which had abundant gene over expression. Cluster 2 had an intermediate level of transcript over expression. Respectively, clusters 1, 2 and 3 consisted of 54%, 41% and 29% metastatic cases. High CD8 rank (high rank defined as 75-100) in clusters 1, 2 and 3 was seen in 15%, 0% and 52% of cases, respectively. Low CD8 rank (low rank defined as 0-24) in clusters 1, 2 and 3 was seen in 69%, 55% and 4.5% of cases, respectively. Clusters 1, 2 and 3 consisted of 92%, 66% and 52% non-UPS, respectively. In clusters 1, 2 and 3 there were 8%, 24% and 38% cases with PD-L1 IHC positivity, respectively (Figure 5).

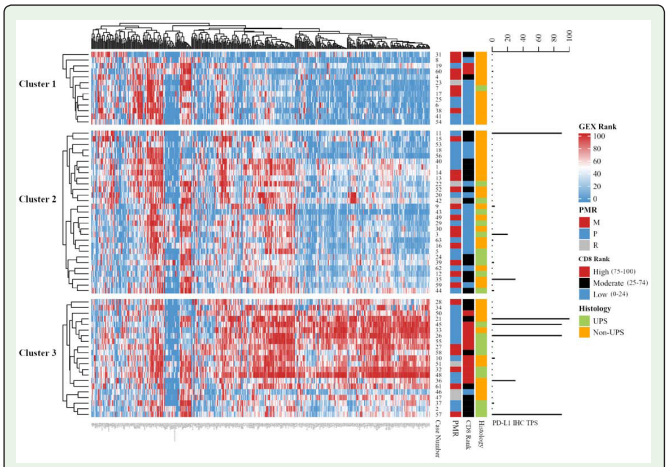

Figure 5: Heat map of 63 sarcomas based on unsupervised clustering.

Discussion

Most of the immunotherapeutic focus in sarcoma has centered on the PD-1 axis [3,4,15,17,18,5-7,10-14]. The expression of a few alternate immunotherapeutic targets, such as CD276, TGFB1, and TIM3, has been previously described in some sarcoma types [25,28,29]. To identify candidate immunotherapy targets we performed RNA expression comparisons between UPS/non-UPS and a non-sarcoma population.

PD-L1 IHC has yielded inconsistent findings in sarcoma partially because of the inclusion of heterogeneous sarcoma entities in many of the relevant studies [3,4,13-15,5-12]. Using various criteria and antibodies most studies show that 40-82% of UPS have PD-L1 IHC positivity [3,7,10,12]. Consistent with this, our UPS population had PD-L1 positivity in 38% of cases, substantially more than the non-UPS population which was PD-L1 positive in 19% of cases (Supplementary table 1). PD-L1 RNA expression in UPS was not significantly different from non-sarcoma cases and was significantly under expressed in non-UPS (Supplementary Tables 2 and 3). However, PD-L2 emerged as significantly over expressed in UPS (Figure 4, Supplementary Tables 2 and 3). PD-L2 has IHC expression in neoplastic and non-neoplastic cells in multiple neoplasms and its expression correlates with pembrolizumab response in head and neck squamous cell carcinoma independent of PD-L1 expression [30]. Along with the high IHC positivity of PD-L1 in UPS, the over expression of PD-L2 in UPS suggests that the PD-1/PD-L1/PD-L2 axis is an important immune response suppressor in UPS. In turn, this raises the possibility that agents targeting PD-L1 may not be as therapeutically efficacious as agents targeting the PD-1 receptor in UPS and provides a possible reason for the high response rate of UPS to pembrolizumab [9].

As additional putative immunotherapy targets, CD276 and TGFB1 were remarkable in that they were both over expressed in UPS and non-UPS (Figure 4, Supplementary Tables 2 and 3).

CD276 is an immune checkpoint molecule which down regulates T-cells (including CD8 T-cells) [31] and activates numerous cancer-related cellular pathways [32]. It has been observed in numerous carcinomas, mediates neoplastic cell migration and invasion, and its expression usually correlates with poor prognosis in cancer [32]. CD276 is immunohistochemically present in most osteosarcomas, is associated with a poor prognosis and with poor infiltration by CD8 T-cells [33]. Our knowledge of its functional significance in other sarcomas is limited, but its known immunosuppressive function and relatively high expression in both UPS and non-UPS suggests that it may be an important immunosuppressor in sarcoma.

Over expression of the immunosuppressive cytokine TGFB1 has been described in sarcomas [25,28,29]. Importantly, one of the functions of TGFB1 is induction of Epithelial to Mesenchymal Transition (EMT) [34-37]. In both UPS and non-UPS we observed over expression of SNAI2, TWIST and ZEB1 which are important actors in EMT (Supplementary tables 2 and 3) [37]. The elevated expression of multiple EMT-related actors, including the immunosuppressive TGFB1, raises the possibility that EMT is an

immunosuppressive process in sarcoma and that its components are exploitable as immunotherapy targets.

CSF1R was significantly over expressed in UPS but not in non-UPS (Figure 4, Supplementary Tables 2 and 3). CSF1R has a well-known association with macrophages [38] and there is preclinical evidence that its blockade increases CD8 T-cell motility and tumor infiltration [39]. In addition to CSF1R, the elevated expression of the macrophage-related CD68 and CD163 in UPS but not in non-UPS raises the possibility that tumor associated macrophages play a particularly important role in UPS, possibly causing immunosuppression [40].

OX40 was overexpressed in non-UPS but not in UPS (Supplementary Tables 2 and 3). OX40 has a costimulatory effect on effector and immunosuppressive T-cells [41,42] and OX40 ligands are known to have an antitumor effect, with one study showing response of a murine sarcoma model to an engineered OX40 agonist [41].

We did not find TIM3 to be significantly differentially expressed in UPS or non-UPS (Supplementary Tables 2 and 3). This is in conflict with the previously described over expression of TIM3 in the TCGA sarcoma cohort [25]. The reasons for this discrepancy are unclear, although the differences in the specific subtypes of sarcomas included in ours and the TCGA cohort may be an explanation. Notably, other potential mmunotherapy targets such as CTLA4, GITR, ICOS, IDO1, and PD-1 were either under expressed or had no expression difference in UPS and non-UPS *versus* non-sarcoma (Supplementary Tables 2 and 3). No cases were found to have microsatellite instability or elevated TMB, suggesting that they may be rarely useful as markers of immunotherapy response in sarcoma.

Unsupervised clustering of all transcripts for all cases revealed three clusters with cluster 1 having the least overall transcript over expression and cluster 3 the most, a trend which included expression of CD8 and PD-L1 status by IHC (lowest in cluster 1, least "inflamed" or "cold", highest in cluster 3, most "inflamed" or "hot") (Figure 5).The increasing percent of metastatic cases from cluster 3 to 1 suggests that metastatic sarcomas tend to have less infiltration by CD8-expressing cells. The causal relationship between metastatic status and presence of CD8 is currently unclear, although there is preclinical evidence indicating that depletion of cytotoxic CD8 T-cells plays an important role in the proliferation of metastatic neoplastic cells [43]. Cluster 1 consisted almost entirely of non-UPS cases (92% of cases), suggesting that UPS does not group with the "coldest" sarcomas. These findings suggest that RNA-expression-based sarcoma "inflammatory" status can be used to categorize sarcomas and possibly, their clinical behavior. Further clinical studies will elucidate whether this type of profiling is also predictive of immunotherapy response.

This study has numerous limitations. The sample size is small and we were only able to include a smattering of various sarcomas in the non-UPS category. Further studies will need large numbers of each specific sarcoma histology. High RNA expression is not necessarily linked to increased protein expression and we could not distinguish between RNA transcribed in neoplastic cells versus RNA transcribed in non-neoplastic cells. Finally, IHC and functional or clinical studies are needed to confirm our findings.

In conclusion, both UPS and non-UPS have a high rate of PD-L1 IHC positivity. UPS in particular has a very high rate of PD-L1 IHC positivity and high PD-L2 RNA expression which suggests a mechanism of immunosuppression and a possible reason why UPS has a high response rate to pembrolizumab. Using RNA expression profiling we confirmed the overexpression of CD276 and TGFB1 in sarcoma and we identified CSF1R overexpression in UPS and OX40 overexpression in non-UPS. We identified an EMT signature in UPS and non-UPS and a macrophage signature in UPS. Inhibition of EMT and tumor associated macrophages provides further avenues of future immunotherapy development in sarcoma. Finally, an "inflamed" RNA-expression signature associated with the presence of CD8 transcript and PD-L1 IHC positivity, and a "cold" signature associated with metastatic disease and non-UPS, suggests that the tumor microenvironment may play an important role in sarcomas. Whether these RNA-expression signatures can be used to predict immunotherapy response in sarcoma remains to be seen.

Disclosures: APS, PDP, SP, FL, JC, BB, VG, JA, GM, SG and CM are all employees of Omni Seq, Inc. (Buffalo, NY) and hold restricted stock in OmniSeq, Inc.; JC, SG and CM are employees of Roswell Park Comprehensive Cancer Center (Buffalo, NY). Roswell Park Comprehensive Cancer Center is the majority shareholder of Omni Seq, Inc.

Data Access, Responsibility and Analysis: APS and CM had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Consent for Publication: OmniSeq's analysis utilized deidentified data that was considered non-human subjects research under IRB-approved protocol (BDR #073166) at Roswell Park Comprehensive Cancer Center (Buffalo, NY).

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