Clinical response of a patient to anti–PD-1 immunotherapy and the immune landscape of testicular germ cell tumors

Running Title: Anti-PD-1 response and immune landscape in testicular cancer

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Anti–Programed Death 1 (PD-1) is standard immunotherapy for multiple cancers and the expression of one of its ligands, PD-L1, has been described in germ cell tumors (GCTs). Neither the clinical activity of anti–PD-1 nor the incidence of an immunoresponsive tumor microenvironment has been described for GCTs. A patient initially diagnosed with melanoma via fine needle aspiration was treated with one dose of antibody to PD-1. A core needle biopsy was subsequently performed to acquire sufficient tissue for molecular analysis, which led to a change in diagnosis to metastatic embryonal carcinoma. The testicular GCT cohort of The Cancer Genome Atlas (TCGA) was analyzed using a T-cell gene signature associated with benefit from immunotherapy. Primary tumors (N = 134) were categorized as high (T cell–inflamed), medium, or low (non-T cell–inflamed) by their T-cell signature derived from RNAseq data. Anti-PD-1 induced decreases in serum markers and a 33% reduction in tumor volume. Gene expression revealed a T cell–inflamed tumor microenvironment in 47% of testicular GCTs, including seminoma (83%) and non-seminoma (17%) tumor subtypes. Expression of alpha-fetoprotein (AFP) RNA correlated with lack of the T-cell signature, with increasing AFP RNA inversely correlating with the inflamed signature and expression of interferon-γ–associated genes. These data suggest that GCTs can respond to anti–PD-1 and that gene expression profiling supports investigation of immunotherapy for treatment of GCTs.
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Introduction

Testicular germ cell tumors, although one of the most common malignancies of young men, are highly treatable with modern chemotherapy and have cure rates exceeding 95% (1). Germ cell tumors are categorized by histological and biochemical parameters as either being a pure seminoma or a nonseminomatous germ cell tumor (NSGCT) with treatment dictated both by American Joint Committee on Cancer and risk modeling–based assessment (2, 3). Early-stage germ cell tumors respond well to orchiectomy and adjuvant radiation or chemotherapy. After using a risk stratification system for more advanced disease, germ cell tumors are commonly treated with 3-4 cycles of chemotherapy. Though disease refractory to initial chemotherapy can be treated with effective second and sometimes third line chemotherapy (4, 5), progressive chemotherapy is associated with significant toxicity. Long-term sequelae of treatment include second malignancies, hypogonadism, infertility and cardiovascular disease (6). As a result, new treatment strategies and novel drug approaches are needed.

Cancer immunotherapy has undergone a resurgence in recent years with the development of immune-checkpoint blocking antibodies such as anti-cytotoxic T lymphocyte antigen 4 (CTLA4) and anti-programmed death 1 (PD-1)/programmed death-ligand 1 (PD-L1). These approaches have changed clinical paradigms in melanoma, non-small cell lung cancer and clear cell renal cell carcinoma, in which phase III trials have documented improvements in overall survival and an improvement in toxicity profile for most patients (7, 8). In many other tumor types, it is expected that checkpoint immunotherapy will become a part of the treatment armamentarium over a short period of time.

Despite tremendous optimism regarding the clinical efficacy of immune-checkpoint immunotherapy, the majority of patients currently do not respond. Multiple predictive
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Biomarkers for immunotherapy have been put forward, particularly immunohistochemistry for PD-L1 or tumor-infiltrating lymphocytes (TIL). However, each of these is only an aspect of an effective antitumor immune response, in which infiltrating T cells elaborate cytokines such as interferon-γ (IFNγ) that leads to upregulation of immune effector molecules (9). A more robust approach to tumor microenvironmental evaluation may thus be facilitated by gene expression profiling where an active antitumor immune environment has been coined the “T cell–inflamed tumor microenvironment” (10). This phenotype has prognostic significance and is associated with the presence of microenvironmental factors—such as PD-L1, indolamine-2,3-dioxygenase (IDO) and regulatory T cells— as well as with clinical responses to immunotherapeutics, such as antibodies to CTLA-4 and PD-1 (11, 12).

Here we describe the effective treatment of a patient with NSGCT with anti–PD-1 immunotherapy and then describe an analysis of the testicular germ cell tumor (TGCT) cohort of The Cancer Genome Atlas (TCGA). Based on gene expression profiling using a previously described T-cell gene signature (13), we found that a substantial proportion of GCTs are likely to be susceptible to checkpoint immunotherapy. Finally, we identified a potential biochemical mediator of immunosuppression whose potential warrants further investigation in prospective clinical research.

**CASE REPORT**

A 32-year-old Caucasian male developed a palpable mass on the left of his neck without other symptoms. Computed tomography (CT) showed a large confluent nodal mass in the left neck (pretreatment, **Fig. 1A and B**; post-treatment, **Fig. 1C and D**) measuring 7.0 x 7.3 cm as
well as retroperitoneal lymphadenopathy that included an aortocaval node of 2.1 x 1.5 cm and paracaval node of 1.2 x 0.9 cm. A fluorodeoxyglucose-positron-emission tomography scan showed hypermetabolism in the left cervical and retroperitoneal lymph nodes. A fine needle aspiration (FNA) demonstrated a malignant neoplasm of large epithelioid cells with high nuclear to cytoplasmic ratio, large and irregular nuclei, and coarse chromatin pattern. Immunohistochemical staining revealed likely positivity of tumor cells for PanMelanoma Cocktail (Mart-1, HMB 45, and tyrosinase) and focal weak staining for pancytokeratin (Fig. 2A-C). These findings were deemed consistent with metastatic malignant melanoma of unknown primary.

The patient was then referred to medical oncology. No obvious skin lesions were observed by physical exam, while baseline laboratory values were within normal limits with the exception of a lactate dehydrogenase (LDH) concentration of 461 U/L (normal range < 200 U/L). The patient began anti–PD-1 therapy with nivolumab, receiving a single 3 mg/kg dose. For consideration of all potential melanoma treatment options, a core needle biopsy was performed to obtain adequate tissue for molecular analysis. In contrast with the FNA, histological review of the core biopsy specimen was consistent with NSGCT (embryonal cell carcinoma) with immunohistochemistry positive for cytokeratin (OSCAR antibody), CD30, and OCT 3/4 (Fig. 2D-F) and negative for S-100, MART-1 (Fig. 2G), SOX10, PLAP, and TTF1/Napsin. Ultrasonography of the scrotum revealed no definitive radiographic evidence of a primary testicular tumor, however the right testicle was markedly atrophic compared with the left, a possible indication of an occult primary tumor. The initial serum tumor markers, measured after nivolumab treatment, included concentrations of α-fetoprotein (AFP) of 1.3 ng/ml (normal range < 8.4), β-human chorionic gonadotropin (HCG) of 3.1 IU/L (normal range < 1.4), and LDH of
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388 U/L. Expression of PD-1 and PD-L1 were determined by a commercial vendor (Pathline Progressive Pathology, Ramsey, NJ). TIL were described as high positive staining for PD-1 with > 25% distribution and 2+ staining intensity, as well as low positive staining for PD-L1 on TIL with 1-24% distribution and 1+ staining intensity. Tumor cells were described as low positive staining for PD-L1 with 1-24% staining in cytoplasmic and membranous location and 1+ intensity.

A follow up CT scan after treatment with a single dose of nivolumab noted the left supraclavicular mass measuring 5.3 x 5.1 cm, aortocaval node 1 x 0.7 cm and paracaval lymph node 0.5 x 0.5 cm, consistent with 33% disease reduction by Response Evaluation Criteria In Solid Tumors version 1.1 and 49% regression by immune-related Response Criteria (14). On clinical exam, the lymphadenopathy in the neck was no longer palpable.

Despite evidence for a rapid response to immunotherapy, tumor board consensus was to transition the patient to standard chemotherapy with bleomycin (30 units/dose, days 1, 8, and 15), etoposide (100 mg/m², days 1 to 5), and cisplatin (20 mg/m², days 1 to 5) administered on a 21-day cycle for 3 cycles, given the curative intent of that approach. After chemotherapy a radiographic complete response was documented and further decline in tumor markers was observed (LDH 263 IU/L, AFP 3.1 ng/mL, HCG < 0.6 IU/L). The patient subsequently underwent right orchiectomy and left neck dissection with evidence of complete pathological response. As the retroperitoneal lymphadenopathy had also resolved, resection of these sites was avoided consistent with professional society guidelines (15).

METHODS
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Analysis of TCGA data set

RNA-seq gene expression data, whole exome sequencing somatic mutation data and clinical information of The Cancer Genome Atlas (TCGA) Testicular Germ Cell Tumors (TGCT) were obtained from Broad Genome Data Analysis Center (GDAC) (release date 11/01/2015, level 4). A total of 134 primary tumor samples were included in this study. RNA-seq raw read counts were summarized at gene level using the expectation maximization (RSEM) method, followed by upper quartile-normalization and log2 transformation.

Identification of T cell–inflamed and non-T cell–inflamed groups

The tumor groups were identified by our previously published method (13). In brief, unsupervised hierarchical clustering was used to cluster 18,475 genes that are expressed in at least 50% of the samples into 12 groups based on similar expression patterns. A cluster of 1,015 genes including 12 genes of a previously identified T-cell gene signature (CD8A, CCL3, CCL4, CXCL9, CXCL10, ICOS, IRF1, GZMK, HLA-DMA, HLA-DMB, HLA-DOA, HLA-DOB) was selected for identification of T cell–inflamed, intermediate and non-T cell–inflamed tumor groups using a consensus sample clustering method. Gene ontology (GO) enrichment analysis of the genes of interest was performed using DAVID v6.7 (16).

AFP expression and correlation with interferon-γ associated immune genes

Tumors from TCGA were divided into five categories based on the expression of Alpha-Fetoprotein (AFP). Samples with 0 raw read counts (AFP negative) were defined as no expression (category 1). For those that express AFP gene (AFP positive), the 1st, 2nd, 3rd, and 4th quartile of normalized AFP expression values (Q1 to Q4, equivalent to 25%, 50%, 75% and 100% expression).
100% percentile) were calculated and used to bin the samples to categories 2 to 5. The distribution of T cell–inflamed and non-T cell–inflamed groups was calculated in each AFP positive or negative category and vice versa. Seminoma and non-seminoma tumor types were retrieved from the patient histological type in the TCGA clinical table. The Pearson’s product-moment correlation coefficient was calculated between AFP and genes of interest. Significance of the association ($P$ values) was computed using an R function correlation test, with the alternative hypothesis being expecting “less” negative association between AFP and select genes.

RESULTS

To better understand the spectrum of IFNγ-associated T-cell inflammation in GCTs, and by extension the relative likelihood that T cell–based checkpoint immunotherapies will have clinical utility, we analyzed of the gene expression data from the TGCT cohort of TCGA, separating patient tumors into T cell–inflamed, intermediate, and non-T cell–inflamed cohorts. The list of TCGA samples and related information is provided in Supplementary Table 1.

Spectrum of T cell–inflamed and non-T cell–inflamed germ cell tumors

By clustering the 134 TGCT samples of TCGA based on the expression of select 1,015 genes (Supplementary Table 2), we identified three distinct tumor groups associated with low (non-T cell–inflamed), high (T cell–inflamed), and mixed (intermediate) expression of the T-cell gene signature (Fig. 3). Overall, the presence of the T cell–inflamed tumor microenvironment was identified in 47% of TGCT and the non-T cell–inflamed tumor microenvironment in 32% (with 21% intermediate). Gene Ontology analysis suggested that expression of these genes is
significantly enriched in biological processes that include immune responses, defense responses, positive regulation of immune system processes, and leukocyte activation (Supplementary Table 3).

**AFP expression inversely correlates with a T cell–inflamed tumor microenvironment**

To explore the role of the T cell–inflamed tumor microenvironment in GCT we evaluated clinical factors, including non-seminoma and seminomatous histology. In the seminoma population, 75% of samples were T cell–inflamed, 19% intermediate, and 6% non-T cell–inflamed. In contrast, within non-seminoma a majority of samples were non-T cell–inflamed at 60%, with 23% intermediate, and 17% inflamed.

Based on the finding of a higher percentage of T cell–inflamed phenotype in seminoma compared to that of non-seminoma, the relative impact of AFP expression in the T cell–inflamed tumor microenvironment was investigated. We categorized AFP expression into 5 levels, from no expression through 4 quartiles, and calculated the relative abundance of each category within the overall T cell–inflamed, intermediate, and non-T-cell inflamed groups. T cell–inflamed tumors with no AFP expression made up 51% of the samples, with another 26% having 1st quartile AFP expression. Conversely, the non-T cell–inflamed population was made up of 35% 3rd quartile and 25% 4th quartile AFP expression. The second quartile AFP expression was similar across inflamed, intermediate, and non-inflamed tumors at 14%, 14%, and 19%, respectively. Distribution of AFP expression by quartile is shown relative to tumor groups in Fig. 4A. Principal component analysis confirmed associations of no/low AFP cohorts with T-cell inflammation and high AFP cohorts with a lack of T-cell inflammation, with 77% of T cell–inflamed tumors having no/low AFP expression (no AFP: 51%; 1st quartile AFP: 26%), whereas
60% of non-T cell–inflamed tumors (3rd quartile AFP: 35%; 4th quartile AFP: 25%) had high AFP expression. Additional analysis revealed statistically significant inverse correlations between AFP and IFNγ-associated genes including CD8A, GZMB, PD-L1, FOXP3, BATF3, and IRF8 (Fig. 4B). A similar analysis was performed on HCG subunit genes (CGA, CGB1, CGB2, CGB3, CGB5, CGB7, and CGB8) with no significant associations observed with T-cell inflammation.

**DISCUSSION**

Here we report a RECIST quality partial response to anti–PD-1 immunotherapy in a patient with advanced NSGCT. This case suggests that further investigation of immunotherapy in this disease is warranted. Our data analysis and assessment were hampered by the inadequate availability of pre-treatment tissue for histologic diagnosis. Therefore, it is clearly a necessity for robust and high quality tissue biopsy specimens to be collected and preserved for later analysis, before initiation of anticancer treatments.

Building upon our clinical case report describing effective treatment of a patient with NSGCT with anti–PD-1, we analyzed the presence or absence of a T cell–inflamed tumor microenvironment of TGCT utilizing an IFNγ-associated gene set. Overall, 47% of TGCT had the T cell–inflamed tumor microenvironment and 32% had the non-T cell–inflamed tumor microenvironment. The T cell–inflamed tumor microenvironment was more likely in seminomatous TGCT and became less frequent in NSGCT as AFP expression increased. Regarding AFP specifically, its expression inversely correlated with several known molecular mediators of immunotherapy.
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Immunotherapy with checkpoint blocking approaches, especially antibodies to PD-1 and PD-L1, has changed the standard of care for several tumor histologies and is being investigated in many cancers. Multiple biomarkers are being pursued to find those that can predict benefit from checkpoint-blockade immunotherapy, including expression of PD-L1 (17) and the presence of infiltrating TIL (18), although both of these are components of an effective IFNγ-associated T-cell response. Thus, gene expression profiling has the potential to be a more encompassing modality, because it reflects the tumor–immune system interaction more broadly.

Despite high cure rates for TGCT, investigation into the immune response associated with these diseases is of increasing urgency given the unmet needs of refractory patients and the long-term toxicities associated with chemotherapy (19). Immunohistochemical analysis for PD-L1 expression has been reported to be present in 73% of seminomas, 64% of non-seminoma, and no staining in intratubular germ cell neoplasia or normal testicular specimens (20). Substantial stromal expression of PD-L1 was also reported, though not in direct comparison with corresponding malignant cells. The presence of PD-L1 expression by immunohistochemistry has been suggested as a prognostic marker in TGCT with a correlation between low PD-L1 expression and improved progression-free and overall survival to non-immunotherapy interventions (21). A previous bioinformatic investigation of TCGA suggested high PD-L1 and CD8A expression in 48% of TGCT samples (22). This is in line with our observations using a T-cell gene signature. Previously we presented preliminary data on an extended 160 gene T-cell signature across TCGA, describing the TGCT cohort as one of the most highly T cell–inflamed, based on percentage of samples with that signature among solid tumors (as in other PD-1 blockade–responsive cancers, including clear cell kidney cancer and lung adenocarcinoma) (23). The dual PD-L1 and CD8A approach above also ranks TGCT highly relative to other tumors,
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adding further support to the concept of high T-cell inflammation in TGCT. An additional advantage of our approach, however, lies in the definition of the non-T cell–inflamed tumor microenvironment, in which we can identify factors driving the immune-exclusion phenotype.

The presence of the T cell–inflamed tumor microenvironment was inversely associated with AFP expression. While this observation requires prospective validation, it is of interest to consider in the context of the known clinical management of GCTs in which concentration of AFP can partially guide the selection of chemotherapy. The intersection of chemotherapy and immunotherapy is of relevance in TGCT, given recent reports suggesting the potential for platinum in sensitizing tumors to checkpoint blockade in preclinical models (24) and clinical reports from multiple tumors of preliminary clinical success combining these modalities (25).

We acknowledge limitations to the data analyses we have advanced here. Although presentation of a case report that demonstrates a clinical response to anti–PD-1 in TGCT suggests the potential for clinical benefit, patients are heterogeneous, making extrapolation of the significance of this case to clinical practice difficult. This patient may have had good-risk, platinum-sensitive GCT, which is not the population of GCT patients most in need of novel treatments. We also recognize that the patients comprising the TCGA data were not treated with immunotherapy and do not represent a homogenous population of patients. Therefore, reference to any individual patient clinical outcomes is not possible and will necessitate prospective studies. Additionally, NSGCT are a heterogeneous group of disease histologies and that the use of AFP as a broad marker will require further refinement, particularly the presence of T-cell inflammation in primary testis tumors versus metastatic disease. Given the inferred immune privilege of the testes under physiologic conditions, tumors arising in a different setting could have different properties. We are not aware of research investigating differences in the immune
response between anatomic sites in germ cell tumors, and sample sizes within TCGA were inadequate to address this in a robust manner.

In summary, we have described a RECIST partial response in a patient with NSGCT, via a single dose of anti–PD-1 and have further described the immune landscape of TGCTs. In particular, a T-cell gene signature in seminomatous GCT was enriched relative to NSGCT, and the presence of the T cell–inflamed tumor microenvironment and the expression of AFP were inversely correlated. These findings could have immediate impact for clinical translation, given that they suggest that a substantial fraction of GCT may be amendable to targeting of T-cell inflammation through approaches such as blockade of PD-1. A phase II study of anti–PD-1 in GCT has been launched (Clinicaltrials.gov identifier: NCT02499952) and results are eagerly awaited.
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References

Legends:

Figure 1. CT images of tumor lesion in neck. (A) Before treatment axial view (B) Before treatment coronal view (C) After 1 dose of nivolumab in axial view (D) After 1 dose of nivolumab in coronal view.

Figure 2. Immunohistochemistry from pre-treatment and on-treatment biopsy. All images at 40x magnification. (A-C) Pan-melanoma cocktail pre-treatment biopsy (D) H&E on-treatment biopsy (E) Oct3-4 on-treatment biopsy (F) CD30 on-treatment biopsy (G) Mart1 on-treatment biopsy.

Figure 3. Gene expression heatmap of non-T cell–inflamed, intermediate and T cell–inflamed testicular germ cell tumors from The Cancer Genome Atlas. Genes are on the row and samples on the column. Two heatmap panels are presented. Top smaller panel shows the expression pattern of HCG genes (CGA and CGB 1/2/3/5/7/8). Bottom larger panel shows that of 1,015 select genes used to categorize samples into T cell–inflamed and non-T cell–inflamed tumor groups, with the gene clustering dendrogram shown to the left side. Blue, red and green horizontal bars indicate tumor groups associated with low (non-T cell–inflamed), high (T cell–inflamed), and mixed (intermediate) expression of the T-cell gene signature. Three horizontal annotation bars are added above the heatmap (from top to the bottom): consensus cluster assignment of 134 primary tumors (clusters 1 to 12); samples colored by AFP expression levels; seminoma (red) and non-seminoma samples (black).
Figure 4. Distribution of AFP expression quartiles within non-T cell–inflamed, intermediate and T cell–inflamed tumor groups. (A) Samples were categorized into five bins based on the expression of AFP: (1) no expression (AFP-); (2-5) 1st, 2nd, 3rd, and 4th quartile of AFP expression level (Q1-Q4, AFP+). (B) Correlation plots of AFP vs CD8A, GZMB, PD-L1, FOXP3, BATF3 and IRF8. P values of the Pearson’s product-moment correlation are indicated above each panel. Blue and red colors indicate the non-inflamed and inflamed tumor group, respectively.
Figure 4

A

B

CD8A
Pearson < 0.0001

GZMB
Pearson = 0.0002

PD-L1
Pearson = 0.0034

FOXP3
Pearson < 0.0001

BATF3
Pearson < 0.0001

IRF8
Pearson < 0.0001

AFP
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