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Differential immune profiles distinguish the mutational subtypes of gastrointestinal stromal tumor

Gerardo A. Vitiello,1 Timothy G. Bowler,2 Mengyuan Liu,1,3 Benjamin D. Medina,1 Jennifer Q. Zhang,1 Nesteene J. Param,1,3 Jennifer K. Loo,1 Rachel L. Goldfeder,4 Frederic Chibon,5 Ferdinand Rossi,1,3 Shan Zeng,1 and Ronald P. DeMatteo1,3

1Department of Surgery and 2Department of Medicine, Memorial Sloan Kettering Cancer Center (MSKCC), New York, New York, USA. 3Department of Surgery, University of Pennsylvania, Philadelphia, Pennsylvania, USA. 4Genome Technologies, The Jackson Laboratory for Genomic Medicine, Farmington, Connecticut, USA. 5INSERM U1037, Cancer Research Center of Toulouse, Toulouse, France.

Gastrointestinal stromal tumor (GIST) is the most common human sarcoma, frequently characterized by an oncogenic mutation in the KIT or PDGFRA gene. We performed RNA sequencing of 75 human GIST tumors from 75 patients, comprising what we believe to be the largest cohort of GISTs sequenced to date, in order to discover differences in the immune infiltrates of KIT- and PDGFRA-mutant GIST. Through bioinformatics, immunohistochemistry, and flow cytometry, we found that in PDGFRA-mutant GISTs, immune cells were more numerous and had higher cytolytic activity than in KIT-mutant GISTs. PDGFRA-mutant GISTs expressed many chemokines, such as CXCL14, at a significantly higher level when compared with KIT-mutant GISTs and exhibited more diverse driver-derived neoepitope:HLA binding, both of which may contribute to PDGFRA-mutant GIST immunogenicity. Through machine learning, we generated gene expression–based immune profiles capable of differentiating KIT- and PDGFRA-mutant GISTs, and identified additional immune features of high–PD-1– and –PD-L1–expressing tumors across all GIST mutational subtypes, which may provide insight into immunotherapeutic opportunities and limitations in GIST.

Introduction

Gastrointestinal stromal tumor (GIST) is the most common sarcoma (1), often originating in the stomach or the small intestine. GIST is a paradigm for targeted molecular therapy, since the majority of GISTs contain an activating mutation in exon 11 of the KIT proto-oncogene, for which there are effective and well-tolerated tyrosine kinase inhibitors (2, 3). Imatinib mesylate, which inhibits the KIT oncoprotein, improved median survival in patients with advanced and metastatic GIST from 1 to 5 years (3), while adjuvant imatinib improved recurrence-free survival in patients with resectable disease (4).

PDGFRA-mutant GIST is the second most common form of GIST. Approximately half of PDGFRA-mutant GISTs contain a D842V substitution, which is inherently resistant to imatinib therapy (5). The majority of PDGFRA-mutant GISTs develop in the stomach (6, 7), while KIT-mutant GISTs can arise in the stomach, small intestine, or rectum (8), suggesting an innate and important biologic difference between these driver mutations. Other GIST mutation types also associate with predictable clinicopathologic features. For example, KIT exon 9–mutant GISTs nearly always develop in the small intestine (9); neurofibromin 1–mutant (NF1-mutant) GISTs are more commonly found in the duodenojejunal flexure (10); and succinate dehydrogenase–deficient (SDH-deficient) GISTs are indolent, multifocal, and among the few GISTs — along with those driven by kinase fusions — that metastasize via the lymphatic system (11, 12). The underlying biologic mechanisms linking mutation type and clinicopathologic features are not well understood.

Although PDGFRA D842V (D842V)-mutant GISTs do not respond to imatinib, natural history shows that recurrence-free survival is more favorable in patients with any PDGFRA mutation when compared with a KIT mutation (4). This finding, along with the predictable behavior of GIST mutation types, had us hypothesize that the mutational driver may impact other aspects of tumor biology, specifically the tumor microenvironment and host immune response. Our group has extensively characterized the immune infiltrate in Kit-mutant GIST using a genetically engineered mouse model and human specimens (13–17), providing a clear rationale for multiple immunotherapy trials in patients with GIST, but the immune response to PDGFRA-mutant GIST is currently unknown.

In this study, we performed RNA-Seq on 75 surgical specimens from 75 patients with GIST to characterize the immune infiltrates of different GIST mutations. Surprisingly, we discovered that PDGFRA-mutant GIST harbors more immune cells than KIT-mutant GIST; this may be related to oncogene-specific cytokine production or more HLA-diverse neoepitope recognition, suggesting that patients with PDGFRA-mutant GIST might benefit from therapeutic immunomodulation. We trained a random forest machine learning algorithm on RNA-Seq data from all of our KIT- and PDGFRA-mutant GISTs, as well as on a subset of untreated, primary, gastric (UPG) KIT- and PDGFRA-mutant GISTs, in order to characterize a PDGFRA-specific immune land-
s ect. Finally, we identified the top immune features correlating with high PD-1 and PD-L1 expression across all GIST specimens to identify potential barriers to and synergistic opportunities for successful immune checkpoint blockade in GIST.

Results

Single-sample gene set enrichment analysis identifies immune cell pathway enrichment in PDGFRA-mutant GIST. We performed RNA-Seq and principal component analysis (PCA) of 75 human GIST specimens comprising various mutation types \( n = 37 \) KIT-mutant, \( n = 24 \) PDGFRA-mutant, \( n = 7 \) SDH-deficient, \( n = 4 \) multiple drivers, \( n = 2 \) WT, and \( n = 1 \) NFI-mutant; Supplemental Table 1; supplemental material available online with this article; https://doi.org/10.1172/JCI124108DS1). The combined PCA of all mutational drivers demonstrated clustering by mutational subtype (Supplemental Figure 1, top), which was most apparent in PDGFRA-mutant and SDH-deficient GISTs (Supplemental Figure 1, bottom). Notably, KIT-mutant GISTs appeared to cluster into 3 distinct groups, which upon closer inspection of clinicopathologic features was related to treatment status and tumor location.

Given what our group has discovered regarding the immune infiltrate of GIST (13–15, 17), the metabolic characteristics of imatinib-treated GIST (18), and the association of cell cycle activity with GIST aggressiveness (19, 20), we used our sequencing data to perform single-sample gene set enrichment analyses (ssGSEAs) focused on immune, metabolic, and cell cycle pathways (Figure 1). Using published gene sets (21), along with assessment of previously published metabolic, cell cycle, and immune pathways (22), we identified 136 gene sets for inclusion in ssGSEA (Supplemental Table 2).
infiltration and cytolytic activity (Figure 1). Meanwhile, SDH-deficient, NFI-mutant, D842V-mutant GISTs with a concurrent CDKN2A (p16) deletion, and WT GISTs (defined as non-KIT, non-PDGFRA, non-RAS-activated, and non-SDH-deficient, as previously described; ref. 25) exhibited generally low ESTIMATE and CYT scores and a lack of immune cell pathway enrichment.

Conversely, ESTIMATE, CYT, and ssGSEA identified increased immune cell infiltration, greater immune cell activity, and a significant enrichment of immune-related gene sets in PDGFRA-mutant GISTs — an unexpected finding that suggested that PDGFRA-mutant GISTs were more immunogenic than other GIST mutations (Figure 1). Cell cycle activity pathways did not clearly correlate with immune cell infiltration across all GIST driver mutation types, while metabolic activity appeared to inversely correlate with the quantity of immune infiltrate, specifically in KIT exon 11-mutant GISTs. Overall, these data show that while tumors driven by a particular GIST mutation can have variable immune-related genes. UPG PDGFRA-mutant GISTs were more commonly found in stomach when compared with KIT-mutant GISTs, and there were significantly more males in our PDGFRA-mutant GIST cohort. There was also a trend toward decreased mitotic activity in PDGFRA-mutant when compared with KIT-mutant GISTs (Supplemental Table 3; n = 61). Though not associated with immune cell infiltration in GIST, tumor size, mitotic rate, and tumor location are predictive of GIST aggressiveness and recurrence-free survival after surgical resection (26). PDGFRA-mutant GISTs with a concurrent KIT-mutant GIST harbored significantly more CD45+ immune cells than KIT-mutant GISTs (Figure 2E). Moreover, flow cytometric analysis of KIT- and PDGFRA-mutant GIST specimens confirmed that PDGFRA-mutant GISTs harbored significantly more CD45+ immune cells than KIT-mutant GISTs (Figure 2E).

CIBERSORT and differential gene expression analysis identify unique immune signatures in GIST. To discover additional differences in immune cell infiltrate between PDGFRA- and KIT-mutant GISTs, we performed IHC staining for CD45 and CD8 on KIT- and PDGFRA-mutant GISTs, including additional tumors not included in the sequencing cohort (Figure 2D). On gross microscopic examination, it appeared that PDGFRA-mutant GISTs contained more CD45+ and CD8+ cells, with a proportion of immune cells clustered around perivascular structures, a finding that we have found to be associated with adaptive immunity (our unpublished observations). Quantification of CD45 and CD8 IHC staining, including staining of the most infiltrated and cytologically active KIT-mutant GIST in the KIT-mutant cohort (human GIST 203), showed that PDGFRA-mutant GISTs had significantly more CD45+ and CD8+ cells per ×20 high-power field (HPF) than KIT-mutant GISTs (Figure 2E). Together, these findings confirm that in PDGFRA-mutant GISTs, immune cells are more numerous and have higher cytolytic activity than in KIT-mutant GISTs with similar clinicopathologic features.

Differential gene expression (DGE) analysis revealed that 20 of 93 (21.5%) immune-related genes were significantly differentially regulated between UPG KIT- and UPG PDGFRA-mutant GISTs (bold indicates P < 0.05; Figure 3, bottom). Specifically, UPG PDGFRA-mutant GISTs expressed significantly more CCR5, BTLA, CD96, CD48, TNFRSF9, TNFSF8, CCR4, CXCL11, CD4 and CD8 IHC staining, including staining of the most infiltrated and cytologically active KIT-mutant GIST in the KIT-mutant cohort (human GIST 203), showed that PDGFRA-mutant GISTs had significantly more CD45+ and CD8+ cells, with a proportion of immune cells clustered around perivascular structures, a finding that we have found to be associated with adaptive immunity (our unpublished observations). Quantification of CD45 and CD8 IHC staining, including staining of the most infiltrated and cytologically active KIT-mutant GIST in the KIT-mutant cohort (human GIST 203), showed that PDGFRA-mutant GISTs had significantly more CD45+ and CD8+ cells per ×20 high-power field (HPF) than KIT-mutant GISTs (Figure 2E). Together, these findings confirm that in PDGFRA-mutant GISTs, immune cells are more numerous and have higher cytolytic activity than in KIT-mutant GISTs with similar clinicopathologic features.

CIBERSORT and differential gene expression analysis identify unique immune signatures in GIST. To discover additional differences in the immune landscape between UPG PDGFRA- and UPG KIT-mutant GISTs, we profiled a previously defined set of 117 relevant immune features (Supplemental Table 5) (28, 29). In addition to ESTIMATE and CYT scores, this profile included scores derived from CIBERSORT, which infers various immune cell frequencies through RNA-Seq deconvolution, as well as 93 relevant immune-related genes. UPG PDGFRA-mutant GISTs appeared to have higher proportions of intratumoral CD4+ T cell and macrophage expression (Figure 3, middle), while UPG KIT-mutant GISTs primarily expressed CD4 and macrophage signatures only in the most immune cell–infiltrated tumors. Furthermore, B cell and CD8+ T cell signatures were also expressed in GISTs with high ESTIMATE and CYT scores, confirming what we and others have shown regarding the immune cell prevalence in GIST (14, 27).

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CXCL14, the mRNA CXCL14 is particularly intriguing. First, KIT-mutant tumors expressed significantly more TNFSF18 and MICA. Together, these data suggest that PDGFRα- and KIT-mutant GISTs elicit a different quality of immune infiltrate, which may ultimately provide driver-specific strategies for immunotherapy.

PDGFRα- and KIT-mutant GISTs have distinct signaling and cytokine signatures. To determine whether intracellular signaling or cytokine differences could be contributing to the observed differences in immune infiltration between UPG PDGFRα-mutant and UPG KIT-mutant GISTs, we utilized GSEA to detect differences in these pathways. On GSEA, we first observed enrichment of a known PDGFRα-mutant GIST signaling pathway gene set from a prior report analyzing 3 PDGFRα-mutant GISTs (Figure 4A, top) (30). Compared with UPG KIT-mutant GISTs, UPG PDGFRα-mutant GISTs also demonstrated significant transcriptional activation of the PI3K signaling pathway (Figure 4A, bottom), which may have been driven by expression changes in PDGFRα, RELN, KDR, or ERK, 4 genes that have been found to be significantly differentially expressed between PDGFRα- and KIT-mutant GISTs not only in our cohort but also previously (30). Furthermore, many cytokine and chemokine pathways were significantly upregulated in UPG PDGFRα-mutant GISTs (Figure 4B), which we found to be related to increased expression of CXCL14, CCL7, CCL19, VEGFA, and KITLG (Figure 4C).

The differences observed in CXCL14 expression were particularly intriguing. First, CXCL14 mRNA was expressed at significantly higher levels in nearly all UPG PDGFRα-mutant GISTs. Quantitative real-time PCR (qRT-PCR) of bulk human PDGFRα- and KIT-mutant tumors also revealed that PDGFRα-mutant GISTs produced approximately 10 times more CXCL14 mRNA than KIT-mutant tumors, and 21 times more CXCL14 mRNA than the KIT-mutant GIST-T1 (Figure 4D, left) and GIST-828 cell lines (data not shown). Finally, CXCL14 has been shown to contribute to NK, CD4+, CD8+, and regulatory T cell chemotaxis and tumor regression in other tumor models (31, 32). UPG PDGFRα-mutant GISTs expressed CXCL14 mRNA at levels higher than those shown to lead to immune-mediated tumor regression in HPV head, neck, and cervical cancers, while KIT-mutant tumors expressed CXCL14 mRNA at levels below this threshold (Figure 4D, right). Together, these findings suggest that CXCL14 expression may contribute to the observed differences in immune infiltration between PDGFRα- and KIT-mutant GISTs (31, 32).

PDGFRα mutation produces multiple HLA-diverse, strong binding neoepitopes. Given the association of neoantigen presentation with inflamed tumor/immune microenvironments in other malignancies, we hypothesized that PDGFRα-mutant GISTs may produce more potent neoepitopes when compared with KIT-mutant GISTs. To explore this, we first mapped out the 8-, 9-, and 10-mer neoepitopes produced by all driver mutations in our cohort, generating neoepitopes with the mutation placed at each amino acid position in the mutant peptide. Then, we tested the binding affinity of each neoepitope to the matched, patient-specific HLA type in which that mutation was found.

First, we wanted to determine whether total neoepitope burden and the number of high-affinity neoepitopes produced by each GIST specimen correlated with immune infiltration or cytolytic activity in GIST, since neoepitope burden has been shown to correlate with increased response to immunotherapy in a variety of cancers (33, 34). However, total neoepitope burden and the number of high-affinity (<500 nM binding) or very high-affinity (<50 nM binding) neoepitopes did not correlate with immune infiltration or cytolytic activity across all mutations in the GIST cohort (Figure 5A) or across KIT- and PDGFRα-mutant GISTs specifically (data not shown).

We observed that the majority of patients with a oncogenic mutation in PDGFRα or KIT produced at least one high-affinity neoepitope (<500 nM binding), regardless of mutation subtype (Figure 5B). We also found that the D842V mutation produced 34 unique, high-affinity neoepitope:HLA-specific binding combinations across the predicted HLA types in our cohort of 16 D842V-mutant patients (Figure 5B), while KIT exon 11 and other PDGFRα mutations averaged produced only 2-3 per mutation (Supplemental Table 6). The D842V mutation generated 6 different neoepitopes that bound to different HLA types with the lowest HLA type in 10 patients in our cohort (Supplemental Table 7), one of which is the most common HLA type in the United States and present in 95.7% of White individuals (HLA-A02:01) (35, 36), suggesting that this mutation could produce an immune response in a wide variety of patients, whereas KIT exon 11 neoepitopes bound to less-prevalent HLA types.

Since the number of patients with D842V mutations (n = 16) exceeded the number of patients with any individual KIT mutation (n = 1–4, Supplemental Table 7), and this may obscure our HLA observations, we explored mutation-specific neoepitope binding diversity more broadly against all HLA types validated by NetMHCPan3.0 (Figure 5C). Again, nearly all PDGFRα and KIT GIST mutations produced at least one high-affinity neoepitope capable of binding to many different HLA types. D842V neoepitopes bound with very high affinity (<50 nM binding) to HLA-A02:01 and many other prevalent HLA types. Interestingly, 50% (4 of 8) of all PDGFRα mutations produced at least one very high-affinity neoepitope (<50 nM binding) to HLA-A02:01, compared with only 14% of KIT mutations (2 of 14). Thus, while all KIT- and PDGFRα-mutant GISTs appear to produce high-affinity neoepitopes, PDGFRα-mutant GISTs appear to produce more high-affinity neoepitopes to more common HLA types.
Given that GIST is often driven by a single oncogenic mutation, and GIST harbors a rich immune infiltrate, this raises the possibility that oncogenic GIST mutations produce neoepitopes that may classify as what has recently been provocatively described as high-quality neoepitopes (37).

Machine learning identifies immune gene signatures for GIST. Through bioinformatics prediction and biologic validation methods, it appears that in PDGFRA-mutant GIST, immune cells are more numerous and have higher cytolytic activity than in KIT-mutant GISTs, and both subsets contain a unique immune pro-
file. This makes characterizing a global immune infiltrate in GIST challenging and suggests that specific immunotherapy approaches may ultimately need to be targeted to the specific oncogenic mutation in GIST. We therefore sought to define the most important immune features differentiating PDGFRA- and KIT-mutant GISTs through machine learning, which is an unbiased approach not dependent on previously defined gene set analysis, allowing for novel and impartial observations. We specifically used all KIT-
Figure 5. PDGFRα mutation produces multiple HLA-diverse, strong binding neoepitopes. (A) Pearson’s correlation of ESTIMATE (top) and CYT (bottom) scores with total neoepitope burden (left), number of high-affinity neoepitopes (middle), and number of very high-affinity neoepitopes (right) among all GIST samples (n = 75). (B) Left: Percentage of patients with the indicated mutation whose mutation produced a predicted high-affinity neoepitope. Right: Number of potential neoepitope:HLA binding events produced by mutation type, averaged over the number of mutations per group. (C) Heatmap of the binding affinities of KIT and PDGFRα mutation-specific neoepitopes to all validated NetMHCPan 3.0 HLA types. Clinicopathologic characteristics of the GIST specimens are shown in Supplemental Table 3. Additional details regarding mutations, neoepitopes, and HLA types used to create this heatmap are shown in Supplemental Tables 6 and 7.
and PDGFRA-mutant GISTs (Supplemental Table 3; \( n = 61 \)), as well as only UPG KIT- and PDGFRA-mutant GISTs (Supplemental Table 4; \( n = 22 \)) to develop 2 random forest models, and assumed that these immune differences would most likely be related to the oncogenic driver.

The combination of salient immune features capable of most accurately profiling a PDGRA- or KIT-mutant GIST were identified based on each feature’s “importance,” which is calculated as part of caret for R’s implementation of randomForest (38). The feature importance metric is a measure indicating how useful a feature is for separating samples into their distinct classes. The features with the highest importance provide the classifier with the highest increase in performance.

We included all 117 immune features to initially create the model (Supplemental Table 5) and subsequently retrained the model using fewer features. We noted that including more features in the model increased the model’s performance on the training set, but decreased the model’s performance on the test set, a phenomenon known as overfitting (Supplemental Figure 3A). Therefore, to prevent overfitting, we retrained the model using only the 6 most important features, which we believe will broaden the applicability of our model to future GIST samples. When we restricted the model to the 6 most relevant features (Figure 6A), 27 of 30 KIT-mutant GISTs were correctly classified as KIT-mutant in the training set, while 14 of 20 PDGRA-mutant GISTs were correctly assigned as PDGRA-mutant (sensitivity: 70%, specificity: 90%). The top immune features included CXCL14, TGFBR1, TNFSF9, MICA, TNFRSF25, and CD96 (Figure 6B). Notably, the PDGRA-mutant tumors that were incorrectly classified as KIT-mutant by our model had lower ESTIMATE and CYT scores than correctly classified PDGRA-mutant tumors (Supplemental Figure 4A), while KIT-mutant tumors incorrectly classified as PDGRA-mutant had more variable CXCL14 mRNA expression and higher TGFBR1 and CD96 mRNA expression than correctly classified KIT-mutant tumors (Supplemental Figure 4B). In our separate cohort of testing samples (\( n = 11 \)), the 6-feature model correctly identified KIT- and PDGRA-mutant GISTs with 91% accuracy (Figure 6C). To demonstrate the importance of these 6 immune features in classifying KIT- and PDGRA-mutant GISTs, we excluded these features and retrained our random forest model, which reduced classifier performance and model accuracy from 91% to 72.7% (Supplemental Figure 3B).

We further validated our model on an external cohort of GISTs from the Complexity Index in Sarcomas (CINSARC) study (\( n = 12 \); ref. 39). Application of our immune profiling model to the CINSARC cohort correctly differentiated KIT- and PDGRA-mutant GISTS with 83.3% accuracy (Figure 6C). Similar results were obtained when we created a model using only UPG PDGRA- and KIT-mutant GISTS (Figure 6, D–F), in which CXCL14, IDO, TNFSF14, KDR, MICA, and TNFRSF9 were the most important features defining the model.

We employed the same machine learning approach to identify gene expression–based immune features associated with high PD-1 and PD-L1 expression across all GISTs (\( n = 75 \)) in order to discover novel therapeutic targets and potential barriers to immune checkpoint blockade in GIST. Interestingly, our 6-feature model showed that increased CD27 expression was highly related to PD-1 upregulation (sensitivity: 90%, specificity: 87.1%, Figure 7A), along with PDCD1LG2, BTLA, CD40, CXCL14, and MICB, suggesting that these pathways may represent synergistic opportunities for PD-1 immunomodulation in GIST (Figure 7B). Using only these 6 features, our model was able to correctly predict high PD-1 expression in 92.9% of the testing cohort and 66.7% of the external CINSARC GIST cohort (Figure 7C).

PD-L1 mRNA expression correlated with PD-L1 protein expression in our cohort (Supplemental Figure 5; see complete unedited gel in the supplemental material). When training our PD-L1 random forest model, expression of key antigen-presenting machinery, including B2M, HLA.DRA, and TAP2, and the immune-responsive cytokines CXCL10 and LTA were predictive of increased PD-L1 expression across all GISTs (sensitivity: 76.7%, specificity: 87.1%, Figure 7D), suggesting a relationship between immune checkpoint ligand upregulation and immune recognition (Figure 7E). Applying the model of these antigen-presenting machinery features and immune cytokines, along with PD-L2, which was also identified as a feature predictive of high PD-L1 expression, our model correctly identified high PD-L1 expression with 85.7% accuracy in the testing cohort and 91.7% in the CINSARC GIST cohort (Figure 7F).

Discussion

The dramatic success of immune checkpoint blockade in a variety of notoriously difficult-to-treat cancers has nearly standardized immunomodulation as an approach for cancer treatment (40–42). However, subsequent research has advanced our understanding of how cancer type– and tumor cell–specific characteristics, such as genomic mutational burden, can predict response to immunotherapy (33, 34). It has also recently been shown that the state of the immune microenvironment can prevent response to immunotherapy if not appropriately addressed (43). In fact, it has become clear that the complex relationship between cancer cell–intrinsic factors and the subtleties of the cancer type–specific immune microenvironment may make a generalized strategy of immune checkpoint blockade ineffective (44). Therefore, it has become increasingly evident that cancer-specific immunotherapeutic strategies are needed.

In this study, we performed RNA-Seq on 75 human GIST specimens from 75 patients to characterize the immune landscape of different GIST mutational drivers, which we believe is the largest cohort of GISTs to be analyzed with next-generation RNA-Seq. After observing potential differences in immune infiltration, we focused on the 2 prevalent GIST mutational subtypes, namely KIT and PDGRA, and found that PDGRA-mutant GISTs contain more immune cells than KIT-mutant GISTS. This suggests that patients with PDGRA-mutant GIST may ultimately have a greater potential to respond to immunotherapy.

Through differential gene expression analysis, we identified mutation-specific immune landscapes that may be significant for directing GIST immunotherapy. First, PDGRA-mutant GISTS showed higher expression of CD96, suggesting that NK cells may play a more substantial role in the immune environment of PDGRA-mutant compared with KIT-mutant GIST. In fact, NK cell infiltrate has been suggested to be a contributor to long-term survival in KIT-mutant GIST (45), which may help explain why PDGRA-mutant GIST patients have a better over-
fore, may be more receptive to a variety of immunotherapeutic approaches, such as targeting TIGIT, BTLA, CD48, or TNFRSF9 in addition to regulatory T cells and NK cells, and PD-1/PD-L1 checkpoint blockade.
Comparison of UPG GISTs of the KIT- and PDGFRA-mutant phenotypes revealed that mitotic rate, an indicator of GIST aggressiveness, was inversely related to the quantity of the immune infiltrate. While this concept has been described in other cancers, tumor proliferative index has not yet been shown to affect the quantity of the immune infiltrate in GIST. PDGFRA-mutant GIST has been shown to have a lower proliferative index when compared with KIT-mutant GIST (46). However, when controlling for mutational driver, the relationship between mitotic rate and immune cell infiltration in UPG GISTs loses significance, suggesting that oncogenic driver may be more important for dictating the immune infiltrate. In fact, comparison of UPG KIT- and PDGFRA-mutant...
GISTs revealed distinct cytokine profiles, which we hypothesize may contribute to immune cell chemotaxis. CXCL14 is a relatively recently identified chemokine with a clear role in immune cell attraction (31, 32, 47). CXCL14 was found to be significantly upregulated in PDGFRα-mutant compared with KIT-mutant GISTs, not only in our cohort but also in a previously published cohort (30). Unfortunately, studying this correlation has proved challenging, as there is currently no PDGFRα-mutant GIST mouse model or human PDGFRα-mutant GIST cell line in which to explore what appears to be a clear biological relationship between PDGFRα tyrosine kinase activation and CXCL14 secretion. Thus, the implication of our finding at this time remains unclear.

It is clear that in cancers with a high number of mutations, tumor mutational burden correlates with immunotherapy response (33). However, the role of neoepitopes in the immune response of cancers with a single oncogenic mutation (i.e., GIST) has not been described. To our surprise, nearly every GIST mutation in our cohort produced a neoepitope capable of binding with high affinity to patient-specific HLA class I, suggesting that even cancers with one mutation can generate a neoepitope recognizable by the immune system. We discovered that the D842V mutation produced 6 different high-affinity neoepitopes, which bound to 12 different HLA types of patients in this cohort alone, one of which is the most common HLA type in the United States and in White individuals (35, 36). While we have not shown that these neoepitopes produced an immune response in these patients, it is intriguing that GISTs with a larger immune infiltrate appear to have a mutation that generates peptides with broad HLA binding specificity, suggesting that this mutation may be of higher quality (37). Moreover, the HLA-specific binding affinities of neoepitopes generated by mutation-specific GIST subtypes reported in this study may provide a road map for patient selection in future neoantigen vaccination trials.

We employed machine learning in order to identify key differences and to profile the expression of immune-related genes between PDGFRα- and KIT-mutant GISTs. Generating a gene expression–based immune profile that accurately predicted the KIT- or PDGFRα-mutant genotype not only supports our hypothesis that the genotype may impact the immune infiltrate, but also introduces another method by which profiling the immune landscapes of KIT- and PDGFRα-mutant tumors can lead to more personalized cancer immunotherapy. Not surprisingly, CXCL14 appeared to be a top feature of PDGFRα-mutant GIST, again suggesting that CXCL14 expression is oncogene specific and may be responsible for the observed differences in immune infiltration and activity. TGFBR1, TNFSF9, MICA, TNFRSF25, CD96, IDO, TNFSF14, KDR, and TNFRSF9 all contributed to the predictive capacity of the immune profiling models, which resulted in >90% diagnostic accuracy in our testing set and in 83% of the CINSARC validation set. We have previously shown the importance ofIDO in dictating the immune phenotype of GIST (13). The role of NK cells and the additional immune modulatory receptors identified in this study should continue to be explored.

Since immunotherapy has not yet shown efficacy in advanced and metastatic GIST (48), we sought to explore the immune profiles of PD-1-high and PD-L1-high GISTs across our entire GIST cohort to identify both therapeutic opportunities and potential barriers to effective immunotherapy. CD27 was found to be an important feature predictive of high PD-1 expression in our random forest model, which correctly identified high PD-1 expression in 93% of our testing GIST cohort. Agonistic CD27 antibodies already exist and have shown efficacy with anti–PD-1 in other models, suggesting that this may be an effective strategy for GIST (49). Similarly, the costimulatory and checkpoint receptors CD40 and BTLA, along with the immune checkpoint ligand PDCD1LG2 and the MHC class I protein MICB were features predictive of high PD-1 expression. We have previously shown that CD40 ligation enhances antitumor immunity in our Kit exon 11 mouse model (17), and these other receptors should be considered in future trials.

Notably, applying our PD-1 model to the external CINSARC cohort resulted in only 67% accuracy, even though our PD-L1 model, in which the same methodology was used, was more accurate (92%). One reason may be related to the small sample size of the external cohort. However, these models identify high-priority targets for further experimental validation.

Finally, expression of key antigen-presenting machinery including B2M, PDCD1LG2, HLA-DRA, and TAP was found to be highly predictive of PD-L1 expression not only in our cohort (86% accuracy) but also in the external CINSARC GIST cohort (92% accuracy), suggesting a tumor cell–specific mechanism of linking neoepitope presentation with immune suppression. A similar finding has been observed in HIV-infected cells, where MHC class I machinery was upregulated concurrently with PD-L1 (50). This is an important concept to consider, as efforts to block PD-L1 may result in tumor cell changes that also downregulate MHC class I antigen presentation, which is essential for the CD8+ T cell–mediated immune response. High PD-L1 expression has also recently been shown to independently correlate with a worse prognosis in soft tissue sarcomas, which was attributed to an increased degree of immune exhaustion, suppression, and negative regulation (51). In contrast, efforts to enhance antigen presentation using neoantigen peptide vaccination or GM-CSF–expressing tumor cell vaccines may require PD-L1 inhibition.

Ultimately, it is becoming increasingly evident that immunotherapy requires a specific approach targeted to not only the tumor type but also the tumor-specific immune microenvironment. Through RNA-Seq and 4 trained machine learning models, we have characterized important and consistent differences in the immune landscape of GISTs driven by different oncogenic mutations, which we believe may help guide future immunotherapy trials in GIST.

Methods

Human GIST specimens and clinicopathologic features. Fresh surgical specimens were collected immediately upon resection and flash frozen in liquid nitrogen. RNA was extracted using the RNeasy kit (QIA-GEN) as per the kit-specified protocol. Clinicopathologic features were obtained via chart review of patient and pathologic records.

Bioinformatics. Poly(A) selection and next-generation RNA-Seq were performed on an Illumina HiSeq 2500 platform by the MSKCC Integrated Genomics Operations core facility. A minimum of 40–50 million 50-bp paired-end reads were obtained for each sample. RNA-Seq reads were processed using Trimomatic v0.36 (52) using the following parameters recommended for paired-end Illumina sequenc-
cross-validation. During each of the 5 folds, a random forest model used to train a random forest machine learning classifier using 5-fold samples were first partitioned randomly into training (80% of sampling: PE - phred33, ILLUMINACLIP: TruSeq3-PE.fa:2:30:10, LEADING: 3, TRAILING: 3, SLIDINGWINDOW: 4:15, and MINLEN: 36). Read counts were then normalized, and DGE analyses were performed on indicated groups using the R software package DESeq2 (54, 55). GSEA was performed using the java GSEA software package (version 3.0) and Molecular Signatures Database (MSigDB) version 6.2 (Broad Institute) (56, 57).

The activity of cytotoxic T cells within each sample was estimated utilizing the CYT score, which was calculated from the geometric mean of the normalized read counts for perforin (PRF1) and granzyme A (GZMA) as previously described (23). The immune cell compartment of these samples was further quantified from the normalized read counts using the R package ESTIMATE (23). The ESTIMATE algorithm is composed of a nonimmune “stromal” parameter and an “immune score” parameter. In this work, we refer to ESTIMATE score as the immune score subcomponent of the ESTIMATE algorithm, which infers the degree of immune cell infiltration within tumor tissue based on expression data of 141 immune-related genes previously shown to correlate with the presence of leukocytes (24). The absolute quantities of individual immune cell subtypes was inferred from the normalized read counts by using the CIBERSORT program (29), which employs an externally validated leukocyte gene signature matrix (LM22) of 547 genes designed to distinguish 22 cell phenotypes including 7 T cell types, B cells, macrophages, monocytes, and NK cells (29). Heatmaps of gene expression data with corresponding clinicopathologic characteristics were created with the R package ComplexHeatmap (58). ssGSEAs were performed using the R software package GSVA (59).

Neoepitopes and HLA prediction. Patient HLA haplotypes were inferred using the program seq2HLA (60). For neoepitope identification, the amino acid sequences of patient-specific mutations were mapped to combinations of 8-, 9-, and 10-mer peptides, with the mutation evaluated at each amino acid position. Each patient-specific peptide was then tested with the respective patient-specific HLA type using NetMHCPan 3.0 to identify potential high-affinity neoepitopes (61, 62). Neoepitope burden was defined as any epitope with a predicted binding affinity within the top 2% when compared with 400 random natural peptides, as recommended by NetMHCPan 3.0. High-affinity neoepitopes were further filtered using a binding affinity threshold of 500 nM, while very high-affinity neoepitopes were defined as having <150 nM binding affinity. Finally, to explore mutation-specific neoepitope binding diversity, we tested mutation-specific neoepitopes more broadly against all validated NetMHCPan 3.0 HLA types and visualized them using heatmap.2 in R.

Machine learning. Random forest modeling with 5-fold cross-validation was performed on the training set using the software package caret for R to identify top predictors (38). One hundred and seventeen features, comprising normalized read counts of various immune genes, and bioinformatically inferred CIBERSORT scores were used to train the model (Supplemental Table 5). For characterization of PDGFRα-specific immune features, KIT- and PDGFRα-mutant GIST samples were first partitioned randomly into training (80% of samples) and testing sets (20% of samples). The training data set was used to train a random forest machine learning classifier using 5-fold cross-validation. During each of the 5 folds, a random forest model was trained on 80% of the training data, and training accuracy, sensitivity, and specificity were calculated; the remaining 20% of the training data were then used to calculate testing accuracy, sensitivity, and specificity. Finally, our trained machine learning model was applied to the 20% testing set and to the external validation CINSARC cohort (n = 12; ref. 39) in order to assess model accuracy on a naive sample set.

Given the total sample size of our cohort and to prevent overfitting of our model, random forest modeling with 5-fold cross-validation was re-performed on the training set using only the top 6 identified features, and the model was then applied to the naive testing set and CINSARC external data set to assess for model accuracy. Feature importance is a variable calculated by the caret package, with a higher number indicating that the feature is more important for model accuracy (38). The confusion matrices shown in Figures 6 and 7 reflect the model, with the highest accuracy after 5-fold cross-validation and optimization by caret. The P value used by caret is calculated using an exact binomial test in which a 1-sided test is performed to determine whether the model accuracy rate is better than the “no information rate,” which represents the proportion of data within the majority class. For generation of PD-1- and PD-L1-specific immune features, the same approach was used, with groups split into high and low expression based on median PD-1 (median: 14.1) or PD-L1 (median: 209.5) normalized read counts in the training set.

qRT-PCR. Total RNA was extracted from snap-frozen human tumors, reverse transcribed, and amplified with PCR TaqMan probe for CXCL14 (Hs01557413_m1). qRT-PCR was performed using a ViIA 7 real-time PCR system (Applied Biosystems). Data were calculated by the 2-ΔΔCt method as described in the manufacturer’s protocol and were expressed as fold increase over the GIST-T1 or GIST-882 cell line control (63, 64).

Flow cytometry. Flow cytometric analysis was immediately performed at the time of specimen collection on freshly obtained human GIST specimens after tumor dissociation as previously described (15). Cells were analyzed on a BD FACSARia or LSRFortessa (BD Biosciences). A human-specific antibody for CD45 (clone D16H11) and PD-L1 (clone EI13N) were purchased from Cell Signaling Technology.

Western blot analysis. Protein from flash-frozen GIST tissue or cell lines was analyzed as described previously (18). Antibodies used against GAPDH (clone D16H11) and PD-L1 (clone EI13N) were purchased from Cell Signaling Technology.

Histology. Freshly collected tumors were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5-μm sections. CD45 (PD7/26 + 2B11) and CD8 (SP57) staining was performed by the MSKCC Molecular Cytology Core. Slides were scanned with MIRAX SCAN (Zeiss) and photographed with Pannoramic Viewer (3DHISTECH Ltd.). For quantification of CD45 and CD8 staining, cell counting was performed manually in a blinded fashion at ×20 magnification (HPF). The number of CD45+ and CD8+ cells per HPF was examined by examining 5 HPFs per tumor and plotting the average per tumor.

Statistics. Some data and graphs were created using Prism 7.0 (Figure 2, A, B, and E; Figure 4, C and D; Figure 5, A and B; Figure 6, B and E; Figure 7, B and E; and GraphPad Software). Statistical tests were performed as described in the figure legends, and when possible, adjusted P values calculated by DESeq2 were used to assess for significance, with a threshold of adjusted P < 0.05. For Figure 7, B and E, an FDR approach was taken using the Benjamini, Krieger, and Yekutieli 2-stage step-up method, with a q value threshold of <0.1 in order to assess for significance.
Data availability and CINSARC external validation GIST cohort. RNA-Seq data presented in this work are available through the Sequencing Read Archive under accession no. PRJNA521803. RNA-Seq data of fresh-frozen GIST tumors from the publicly available CINSARC cohort (39) were obtained from the Sequence Read Archive under accession no. SRP057793.

Study approval. Tumor specimens were obtained from 2011 to 2017 from 75 GIST patients who underwent surgical resection at MSKCC. Prior to resection, patients provided written informed consent under an IRB-approved protocol. Using a prospectively maintained institutional database, clinicopathologic characteristics were documented and encrypted, and all research was conducted in compliance with Health Insurance Portability and Accountability Act regulations.

Author contributions GAV, TGB, BDM, and RPD contributed to study conception and design. GAV, TGB, and RPD contributed to the development of research methodology. GAV, TGB, BDM, JQZ, JKL, NJP, SZ, ML, and FC contributed to the acquisition of data and conduct of experiments. GAV, TGB, ML, RLG, NJP, FR, and RPD contributed to the analysis and interpretation of data. GAV, TGB, and RPD contributed to the writing, review, and revision of the manuscript. All authors reviewed and contributed comments that were incorporated into the final version of this manuscript. Study supervision was provided by RPD.

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Address correspondence to: Ronald P. DeMatteo, 3400 Spruce Street, Philadelphia, Pennsylvania 19104, USA. Phone: 215.662.7539; Email: ronald.dematteo@uphs.upenn.edu.

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