Stratification of Pancreatic Ductal Adenocarcinoma: Combinatorial Genetic, Stromal, and Immunologic Markers

Erik S. Knudsen1,2,3, Paris Vail3, Uthra Balaji1, Hoai Ngo1, Ihab W. Botros4, Vladimir Makarou5,6, Nadeem Riaz5,6,7, Vinod Balachandran8, Steven Leach8, Debrah M. Thompson4, Timothy A. Chan5,6,7, and Agnieszka K. Witkiewicz1,2,3,9

Abstract

Purpose: Pancreatic ductal adenocarcinoma (PDAC) is associated with an immunosuppressive milieu that supports immune system evasion and disease progression. Here, we interrogated genetic, stromal, and immunologic features of PDAC to delineate impact on prognosis and means to more effectively employ immunotherapy.

Experimental Design: A cohort of 109 PDAC cases annotated for overall survival was utilized as a primary discovery cohort. Gene expression analysis defined immunologic subtypes of PDAC that were confirmed in the Cancer Genome Atlas dataset. Stromal and metabolic characteristics of PDAC cases were evaluated by histologic analysis and immunostaining. Enumeration of lymphocytes, as well as staining for CD8, FOXP3, CD68, CD163, PD-L1, and CTLA4 characterized immune infiltrate. Neoantigens were determined by analysis of whole-exome sequencing data. Random-forest clustering was employed to define multimarker subtypes, with univariate and multivariate analyses interrogating prognostic significance.

Results: PDAC cases exhibited distinct stromal phenotypes that were associated with prognosis, glycolytic and hypoxic biomarkers, and immune infiltrate composition. Immune infiltrate was diverse among PDAC cases and enrichment for M2 macrophages and select immune checkpoints regulators were specifically associated with survival. Composite analysis with neoantigen burden, immunologic, and stromal features defined novel subtypes of PDAC that could have bearing on sensitivity to immunologic therapy approaches. In addition, a subtype with low levels of neoantigens and minimal lymphocyte infiltrate was associated with improved overall survival.

Conclusions: The mutational burden of PDAC is associated with distinct immunosuppressive mechanisms that are conditioned by the tumor stromal environment. The defined subtypes have significance for utilizing immunotherapy in the treatment of PDAC. Clin Cancer Res; 23(15); 4429–40. ©2017 AACR.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is therapy recalcitrant and has yet to see significant improvement in long-term survival beyond a few weeks with recent chemotherapy regimens (1–3). Therefore, there is a major opportunity to provide precision approaches to PDAC treatment. Genetic analyses of pancreatic cancer revealed few currently actionable targets for therapeutic intervention (4–6). However, these analyses demonstrated that a subset of PDAC is highly chromosomally unstable or associated with mutator-like phenotypes that could serve as the basis for a targeted approach to the use of immunotherapy in pancreatic cancer. Most notably, a large phase III trial of Algenpantucel-L vaccine recently failed to demonstrate clinical improvement, as did multiple single-agent checkpoint inhibitor trials (14, 15). These findings suggest a need for thoughtful and targeted approach to the use of immunotherapy in pancreatic cancer that will require combinatorial treatments overcoming complex immunosuppressive mechanism and/or increase recruitment of immune cells to the tumor site.

Cancer microenvironment was postulated to limit immune cell infiltration and impair their function in the tumors (16, 17). A unique feature of PDAC is the presence of a desmoplastic stroma...
Translational Relevance

Pancreatic cancer is a therapy-recalcitrant disease for which targeted interventions are needed. Although successful in several solid tumor types, to date, single-agent immunotherapy trials yielded no clinical benefit in pancreatic cancer. This is because mutational burden of PDAC is associated with distinct immunosuppressive mechanisms that are conditioned by the tumor stromal environment. Composite analysis with neoantigen burden, immunologic, and stromal features defined novel subtypes of PDAC that could have bearing on sensitivity to immunologic therapy approaches. These findings suggest a need for thoughtful and targeted approach to the use of immunotherapy in pancreatic cancer that will require combinatorial treatments overcoming complex immunosuppressive mechanism and/or increase recruitment of immune cells to the tumor site.

Materials and Methods

Patient cohort, sequencing, and tissue microarray

Patients with a diagnosis of PDAC were consented for tissue collection and exome-sequencing analysis under an institutional review board (IRB)-approved protocol at the University of Texas Southwestern (Dallas, TX). The 109 patient cohort and sequencing analysis has been previously described although follow-up data was extended. Formalin-fixed paraffin-embedded PDAC tissue was evaluated by an anatomic pathologist for the construction of tissue microarrays. The arrays were constructed using standard approaches.

IHC analysis and staining

Hematoxylin staining and evaluation of the tissue architecture were used to define the stromal type. The TMAs were stained with the following antibodies: PD-L1 (Cell Signaling Technology, dilution 1:600), CTLA4 (Santa Cruz Biotechnology, dilution 1:300), CD163 (Cell Marque, prediluted), FOXP3 (Abcam, dilution 1:200), and CD8 (DAKO, prediluted). The tissues were also stained for CA9 indicative of hypoxia antibody (Cell Marque, dilution of 1:500). MCT4 was employed as a marker of glycolytic preference (Santa Cruz Biotechnology, dilution 1:250). Expression of IHC markers was categorized semiquantitatively using published criteria. Stromal tumor--infiltrating lymphocytes (TIL) were assessed based on criteria developed by Denkert and colleagues (30).

Oncoimmunology panel and neoantigen determination

The HTG EdgeSeq Immuno-Oncology Panel was run on pancreatic FFPE samples as described previously (31). This assay contains probes to measure the expression of 549 RNAs. Briefly, the area of interest was identified on 109 pancreatic FFPE sections (5 μm on glass slides), and was scraped and lysed in HTG’s lysis buffer. Depending on the size of the section, either 7.5 mm² or 15-mm² tissue was used in the assay. Following nuclease protection, each sample was tagged individually with molecular barcodes; tagged samples were pooled and sequenced on an Illumina MiSeq or NextSeq sequencer. Fastq files from the individual samples were processed and the expression data reported by the HTG EdgeSeq parser software. Data from two samples did not pass QC metrics and were excluded from the analysis. Neoantigen analysis was performed as described previously on exome sequencing data (32).

Bioinformatic analysis

Unsupervised hierarchical clustering and Pearson correlation analysis were performed using R. TCGA data was obtained from the TCGA web portal. Gene ontologies were determined using DAVID Functional Analysis tool for enrichment analysis. Network diagrams were generated using ReactomeFIViz (33). Unsupervised random forest clustering, survival analysis and multivariate Cox proportional hazards statistics were also performed using R.

Results

Immunologic expression features of pancreatic cancer

To probe the intersection between tumor genetics, immune system, and stromal features of disease, we interrogated a cohort of 109 surgically resected PDAC cases. The median follow-up was 560 days and the 2-year overall survival was 59%, which is consistent with historic clinical presentation (Supplementary Fig. S1). None of the cases had received neoadjuvant chemotherapy or radiotherapy. Initially, a subset of this cohort was interrogated using HTG EdgeSeq system that applied novel target capture and library preparation chemistry enabling RNA sequencing from FFPE samples (Fig. 1). A panel of 549 genes implicated in host immune response to the tumor was employed to measure tumor immune cell composition, chemokines, and other immunomodulatory soluble factors using a single section of FFPE tissue. These data demonstrated that two principle gene expression clusters could be delineated in PDAC cases (Fig. 1A). The first gene expression cluster that was expressed at a higher level in approximately 50% of cases (demarcated with yellow color-bar) was enriched for 191 genes implicated in modulating lymphocyte activation and proliferation (Fig. 1B). Highly expressed genes in this cluster also included cytokine/chemokine and interleukin...
signaling (Fig. 1C). The second cluster was characterized by lower expression of multiple immune pathways genes; however, it was enriched for adhesion molecules and proteasomes (Fig. 1D). To further characterize the composition of immune pathways within the two clusters, a series of previously published signatures were employed (Fig. 1E, 1F, and S2). These analyses demonstrated that the cases segregated on the basis of enrichment for T-cell and B-cell receptor signaling pathways (yellow cluster) versus macrophage,
tumor-infiltrating lymphocytes (TIL) to tumor cells and creating hypoxic immunosuppressive environment modulating function of recruited immune cells. To investigate impact of PDAC micro-environment on immune composition of the tumor, we initially evaluated stromal volume using tumor hematoxylin and eosin-stained sections. When cases were dichotomized around the median stromal volume, there was a statistically significant association of low stromal volume with poor overall survival (Fig. 2A; Supplementary Fig. S3) as previously reported (34, 35). Next, we evaluated morphologic features of the stromal compartment consistent with the reported biological diversity of stroma (29). When evaluating the number of cancer associated fibroblasts (CAFs), presence of mature collagen fibers and loose stromal matrix, PDAC cases could be divided into three stromal subtypes. These included cases with dense collagenous stroma and low number of CAFs (called “mature”), a highly cellular and collagen-poor stroma (“immature”), and an intermediate form of the stroma (“intermediate”; Fig. 2B). The morphological characteristics of PDAC stromal compartment were strongly associated with prognosis, wherein the immature stroma correlated with shorter overall survival (Fig. 2B).

Metabolic and hypoxic features of pancreatic tumors are related to stromal features of disease

The hypoxia and aberrant production of metabolites in the tumor microenvironment can result in a multitude of effects ranging from preferential recruitment of specific immune cell subtypes to impacting directly on innate and adaptive effector cell functions. CD8+ T cells, although highly cytolytic, are conditioned by the tumor stroma (37–39). The total number of macrophages (TAM) can limit immune engagement locally and have become an exciting target in the treatment of PDAC (39–41). The number of TAMs correlated with stromal type, where immature stroma exhibited significantly increased macrophage content. The expression of CTLA4 was determined in lymphocytes and we observed that the presence of CTLA4-positive cells was associated with poor outcome (Fig. 4B). In contrast, FOXP3, which is a marker of T-regulatory cells, had no relationship to prognosis (Fig. 4B). While there was no association between stromal type and number of FOXp3+ cells, CTLA4 lymphocytes were enriched in the immature stromal type (Fig. 4B). Finally, the expression of PDL1 was evaluated in the tumor cells (PDL1-T), in the immune cells along the invading edge of the tumor (PDL1-Front), and in the tumor microenvironment (PDL1-TME). Only in the tumor microenvironment, PDL1 levels were associated with survival (Fig. 4C; Supplementary Fig. S3). These findings illustrate the complexity of PDAC immune milieu and suggest that in any given tumor multiple and diverse immunosuppressive mechanisms could be at play to impact immunotherapeutic strategies.

Distinct stromal compartments in pancreatic cancer are associated with prognosis

While innate and adaptive immune responses are active during initial stages of PDAC precursor development, immune evasion is a common feature of established PDAC tumors. Previous studies demonstrated that stromal compartment played important role in modulating and dampening immunological responses in PDAC (23). PDAC stromal compartment can impact immune responses via several mechanisms including limiting access of tumor-infiltrating lymphocytes (TIL) to tumor cells and creating hypoxic immunosuppressive environment modulating function of recruited immune cells. To investigate impact of PDAC micro-environment on immune composition of the tumor, we initially evaluated stromal volume using tumor hematoxylin and eosin-stained sections. When cases were dichotomized around the median stromal volume, there was a statistically significant association of low stromal volume with poor overall survival (Fig. 2A; Supplementary Fig. S3) as previously reported (34, 35). Next, we evaluated morphologic features of the stromal compartment consistent with the reported biological diversity of stroma (29). When evaluating the number of cancer associated fibroblasts (CAFs), presence of mature collagen fibers and loose stromal matrix, PDAC cases could be divided into three stromal subtypes. These included cases with dense collagenous stroma and low number of CAFs (called “mature”), a highly cellular and collagen-poor stroma (“immature”), and an intermediate form of the stroma (“intermediate”; Fig. 2B). The morphological characteristics of PDAC stromal compartment were strongly associated with prognosis, wherein the immature stroma correlated with shorter overall survival (Fig. 2B).

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The hypoxia and aberrant production of metabolites in the tumor microenvironment can result in a multitude of effects ranging from preferential recruitment of specific immune cell subtypes to impacting directly on innate and adaptive effector cell functions. MCT4 and CA9 were employed as markers for glycolysis and hypoxia, respectively, due to their well-characterized functions in tumor biology (36), and established conditions for immunostaining to delineate compartment-specific relationship to prognosis. We observed that glycolytic metabolic preference (characterized by high expression of lactate monocarboxylate transporter 4-MCT4) and hypoxia (characterized by high expression of carbonic anhydrase 9-CA9) were associated with poor outcome in this cohort (Fig. 2C and E; Supplementary Fig. S3). These findings are consistent with previously published work in independent patient cohorts (37, 38). To probe the inter-relationship between hypoxia, metabolic features, and stromal volume, the stromal type was used to stratify these parameters (Fig. 2D). These data indicated that the immature stromal type was associated with low stromal volume, and high levels of stromal CA9 and MCT4. Correlation analysis indicated that the stromal variables are related (not shown); however, they all harbor prognostic value above standard pathologic features (e.g., tumor grade, nodal status, and tumor stage; Fig. 2E).

TILs are conditioned by the tumor stroma

The composite features of stromal architecture and metabolism are expected to influence the immune milieu within the tumor. As a first step in analyzing immune features of PDAC, we evaluated the number of TILs in the tumor and in the areas adjacent to tumor (Peri-T lymphocytes). Higher levels of lymphocytes around the tumor were associated with lower overall survival (Fig. 3A). Interestingly, the number of TILs within the stromal tumor compartment was not a prognostic feature (Fig. 3B). Similarly, the number of CD8+ T cells, although highly variable across cases, was not associated with survival (Fig. 3C). To evaluate how stromal biology may impact on the infiltrate, the levels of TILs and CD8+ T cells, cases were stratified on the basis of stromal type and stromal volume (Fig. 3D). Stromal type influenced the abundance of TILs, wherein collagenous mature stroma had lower number of infiltrating lymphocytes. In contrast, stromal type had no impact on the number of CD8+ T cells, suggesting that other subsets of lymphocytes account for a differences in TILs.

Multiple immunosuppressive mechanisms are engaged in pancreatic cancer

Differential immune cell recruitment could be reflective of distinct immunosuppressive mechanisms in the tumor microenvironment. Tumor-associated macrophages (TAM) can limit immune engagement locally and have become an exciting target in the treatment of PDAC (39–41). The total number of macrophages in the tumor environment was determined by CD68 staining. The presence of high number of macrophages was associated with poor prognosis (Fig. 4A). The expression of CD163, indicative of suppressive M2 macrophages, was also associated with decreased survival consistent with other studies (Fig. 4A; ref. 42). Importantly, the number of TAMs was strongly correlated with stromal type, where immature stroma exhibited significantly increased macrophage content. The expression of CTLA4 was determined in lymphocytes and we observed that the presence of CTLA4-positive cells was associated with poor outcome (Fig. 4B). In contrast, FOXP3, which is a marker of T-regulatory cells, had no relationship to prognosis (Fig. 4B). While there was no association between stromal type and number of FOXp3+ cells, CTLA4 lymphocytes were enriched in the immature stromal type (Fig. 4B). Finally, the expression of PDL1 was evaluated in the tumor cells (PDL1-T), in the immune cells along the invading edge of the tumor (PDL1-Front), and in the tumor microenvironment (PDL1-TME). Only in the tumor microenvironment, PDL1 levels were associated with survival (Fig. 4C; Supplementary Fig. S3). These findings illustrate the complexity of PDAC immune milieu and suggest that in any given tumor multiple and diverse immunosuppressive mechanisms could be at play to impact immunotherapeutic strategies.
Immunologic Subtypes of Pancreatic Cancer

Figure 2.
Stromal and metabolic features are associated with prognosis. A, PDAC cases exhibit distinct stromal volume as shown in the representative images. PDAC were stratified based on stromal volume and the association with survival is shown. Data were dichotomized based on established cutpoints described in Materials and Methods. B, PDAC cases exhibit three distinct stromal subtypes as shown in the representative images. Cases were stratified on the basis of stromal type and the immature form of PDAC stroma was significantly associated with poor prognosis as determined by Kaplan–Meier analysis. C, The expression of MCT4 and CA9 are markers of glycolytic and hypoxic environments respectively. The high expression of each marker was significantly associated with poor prognosis as determined by Kaplan–Meier analysis. Data were dichotomized based on established cutpoints described in Materials and Methods. D, The stromal volume, stromal MCT4 expression, or stromal CA9 expression were evaluated dependent on the stromal type. Statistical association was determined by Student t test (*, P < 0.05; **, P < 0.01; ***, P < 0.001). E, Multivariate analysis of the prognostic significance of stromal volume, stromal type, stromal MCT4, or stromal CA9 were determined against the clinical variable grade, tumor stage, and nodal status. Each marker remains significant in the multivariate model.
Figure 3.
Tumor-infiltrating cells and prognosis. A, TILs in the periphery of the tumor were scored on whole tissue tumor section. Data were dichotomized based on established cutpoints described in Materials and Methods. The association with survival was determined by Kaplan–Meier analysis. B, The level of TILs was determined using established criteria by a surgical pathologist with extensive experience in PDAC histology. The level of TILs was not significantly associated with overall survival as determined by Kaplan–Meier analysis. Data were dichotomized based on established cutpoints described in Materials and Methods. C, CD8⁺ cells within the tumor were quantified and exhibited diverse levels across the tumor. The level of CD8⁺ cells was not significantly associated with overall survival as determined by Kaplan–Meier analysis. Data were dichotomized based on established cutpoints described in Materials and Methods. D, The stromal TILs and CD8⁺ cells were evaluated dependent on the stromal volume and stromal type. Statistical association was determined by Student t test (*, P < 0.05; **, P < 0.01).
Figure 4.
Differential engagement of immune suppressive features in PDAC. **A,** The presence of macrophages or type II macrophages was determined by staining for CD68 and CD163, respectively. The overall presence of macrophages within the tumor microenvironment was significantly associated with overall survival as determined by Kaplan-Meier analysis. The level of CD68⁺ and CD163⁺ cells was associated with an immature stromal type. Data were dichotomized based on established cutpoints described in Materials and Methods. **B,** The presence of FOXP3-positive cells (indicative of T regulatory cells) or CTLA4⁺ lymphocytes was determined within the PDAC tumors. CTLA4⁺ lymphocytes were significantly associated with overall survival, while FOXP3 was not significantly associated with overall survival as determined by Kaplan-Meier analysis. The level of CTLA4⁺ and FOXP3⁺ cells was determined as a function of stromal type. Data were dichotomized based on established cutpoints described in Materials and Methods. **C,** The expression of PDL1 was determined by immunostaining both in tumor cells (PDL1-T) and in the tumor microenvironment (PDL1-TME). The association with overall survival was determined by Kaplan-Meier Analysis. The level of PDL1 in various tumor compartments was analyzed as a function of stromal type. Data were dichotomized based on established cutpoints described in Materials and Methods. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. 
Composite relationship of neoantigens and stromal features with immune milieu defines prognostic subtypes of pancreatic cancer

The burden of tumor-specific antigens (neoantigens) that emerge as a product of mutational processes in the tumor was shown to impact responses to immune checkpoint therapy (32, 43). As all cases in the cohort were exome sequenced, the presence of neoantigens was determined using an established computational method (32). PDAC exhibited diverse levels of neoantigens (Fig. 5A); however, the number of neoantigens per tumor was generally lower than that observed in melanoma or lung cancer wherein immunotherapy with checkpoint inhibitors has been most effective (4, 6). Next, correlation analysis that integrated stromal type, immune infiltrate, and neoantigen burden was employed to define landscape features of PDAC. These data revealed the presence of multiple interdependent processes that were generally inversely associated with stromal volume and longer survival (Fig. 4B). We also employed the TCGA data to determine whether observed patterns of expression were preserved in an independent cohort. In the TCGA cohort, gene expression of immunologic markers including PDL1 (CD274), FOXP3, CTLA4, CD8, CD68, and CD163 were positively correlated, and inversely related to survival times (Supplementary Fig. S4).

Unsupervised random-forest clustering using neoantigens, stromal, and immune infiltrate features yielded four distinct “immuno-subtypes” of PDAC (Fig. 4C; Supplementary Fig. S5). Cluster 4 exhibited low levels of veritably all immune and stromal markers, harbored a mature stromal type, high stromal volume, and low number of neoantigens (Fig. 5C and D). While these tumors exhibited the ubiquitous activation of KRAS, they were underrepresented for mutations targeting other canonical genetic events in PDAC (e.g., CDKN2A, MYC, and TP53). This configuration ostensibly represents a “cold” tumor. Cluster 1 also harbored low number of mutations; however, it exhibited high levels of MCT4, low stromal volume, and immature stromal type (Fig. 5C). This finding indicates that glycolytic tumor microenvironment is not universally a feature of high-mutational burden in PDAC. The immune infiltrate in Cluster 1 was dominated by macrophages, likely induced by glycolytic and acidic microenvironment. Cluster 3, harbored a high mutational burden and intermediate morphologic stromal type, higher numbers of TILs and peritumoral lymphocytes, but exhibited relatively low levels of CD68 and CD163+ macrophages. Cluster 2, demonstrated high levels of veritably all immune cell subsets and was also mutationally active. Of the clusters, Cluster 4 was associated with increased overall survival that was significant in univariate analysis with each of the other immune subtypes (Fig. 4E and F). Cluster 4 was also associated with improved outcome when considering tumor grade and lymph node status in multivariate analysis (Fig. 4G). Interestingly KRAS Q61 mutations were enriched for in this cluster (Supplementary Fig. S5) and could contribute to longer survival, as KRAS Q61 mutation was shown to be predictor of better prognosis (4).

Discussion

Immunotherapy holds substantial promise for tumors that are recalcitrant to standard therapies and for which disease recurrence is a major clinical problem. Here, we explored features of the immune system and microenvironment to delineate subtypes of PDAC that may be expected to be responsive to distinct forms of immunotherapy. These data illustrate that there is a profound diversity in the nature of immune response in PDAC that is not solely governed by neoantigen burden, and that select features of the tumor microenvironment are associated with distinct immunosuppressive mechanisms.

The role of the immune milieu of PDAC as a prognostic feature is only starting to emerge. Here, we analyzed multiple different subsets of tumor-infiltrating immune cells. The overall burden of TILs or CD8+ T cells was not associated with overall survival consistent with the notion that PDAC represents “non-immunogenic” tumor (44, 45) and fibroblast activation protein α-expressing CAFs contribute to lymphocyte exclusion in PDAC (26). These findings contrast with “immunogenic” cancers that are characterized by naturally occurring high number of TILs and respond to immunotherapy (30, 46). We did not observe prognostic significance of FOXP3+ regulatory T cells in agreement with recent meta-analysis study (47). In contrast, the presence of macrophages (CD68 positive) and in particular M2 macrophages (CD163 positive) had negative effect on survival. These data are consistent with an emerging literature that the presence of tumor-associated macrophages (TAM) is associated with more aggressive form of disease (40, 48). In addition to supporting tumor invasiveness and metastatic spread, TAMs inhibit T-cell responses by production of indoleamine dioxygenase metabolites and reactive oxygen species and indirectly by recruiting regulatory T cells to the tumor (49, 50). Regulatory T cells in turn inhibit T-cell production of IFNγ and IL2 in response to tumor-specific antigens, as well as their cytotoxic function with resulting impediment to naturally occurring antitumor immunity. Expression of the immunosuppressive proteins CTLA4 and PDL1 was observed in a subset of PDAC with PDL1 expression noted in several compartments. CTLA4 expression on immune cells and PDL1 positivity in tumor microenvironment were significantly associated with poor overall survival. In contrast to melanoma and non–small cell lung cancer, there was no prognostic significance for PDL1 expression in tumor cells or in the immune cells at the tumor-invasive front. That is consistent with the concept that PDL1 expression on tumor cells correlates with number of tumor neoantigens and TILs (51). Interestingly, PDL1 expression in the TME was a predictor of poor survival and associated with immature stromal type and glycolytic metabolic preference. Recent study has demonstrated that depletion of glucose from TME and resultant lactate production can become limiting for T-cell effector functions, as this subset of immune cell is dependent on aerobic glycolysis (52). In contrast, regulatory T cells and macrophages are capable of utilizing fatty acid oxidation to survive in low-glucose environment (53, 54). Together, these data illustrate the diversity of the immune system in PDAC that would ostensibly condition any approaches to immunotherapy.

PDAC is somewhat unique among other solid tumors in having a particularly prominent stromal component that has been proposed to limit vascularization of the tumor and provide a mechanical barrier limiting recruitment of immune cells. The volume of the stromal compartment in PDAC, as well as its histomorphologic characteristics, is highly variable across cases. We observed that immature stromal type (dominated by cancer-associated fibroblasts and poor in mature collagen) was associated with a higher number of TILs and diversity of immune repertoires than collagen-rich mature stroma. This finding supports the hypothesis...
Figure 5.
Composite analysis of tumor genetics, microenvironment, and immune milieu. A, The presence of neoantigens in the tumor cohort was determined from whole-exome sequencing. Graph demonstrates the number of neoantigens per case. B, Correlation analysis between histologic features, the number of neoantigens, the number of mutations, immunologic/metabolic markers, and overall survival is summarized in the heatmap. C, Random-forest clustering was employed on all of the markers summarized in the heatmap to define four clusters (red, high; orange, intermediate; blue, low). The presence of hallmark genetic alterations targeting KRAS, CDKN2A, SMAD4, TP53, and MYC are shown in the color bar (green, mutation; orange, INDEL; red, amplification; blue, deletion). D, Quantification of the number of neoantigens in each of the clusters is shown (**, \( P < 0.01 \)). E, The association of the random forest clusters with survival was determined by Kaplan-Meier analysis and statistical significance was assessed by log-rank analysis. F, The analysis of Cluster 4 versus all other cases was determined by Kaplan-Meier analysis and statistical significance was determined by log-rank analysis. G, The significance of the random forest clusters was evaluated by multivariate analysis relative to grade and lymph node (LN) status. Cluster 4 remained significant relative to improved outcome.
that in some cases dense desmoplastic stroma may provide a mechanical impediment to recruitment of immune cells. Interestingly, immature stroma was also associated with hyposia and a more glycolytic metabolism. The acidic pH has been shown to suppress CD8+ T-cell effector activity and recruit macrophages and in our cohort we could observe the association between increased expression of lactate exporter MCT4 and higher number of CD68+ macrophages and CD163+ M2 macrophages (55).

The engagement of antitumor immunity is currently believed to be conditioned by the number of neoantigens that represent mutated peptides that are shed from tumor cells and considered as non-self (43). Consistent with this overall hypothesis, tumors that have very high mutational burdens have been found to elicit more of an antitumor immune response and represent disease for which immune checkpoint inhibitors appear to be effective (e.g., melanoma, non–small cell lung carcinoma; ref. 32). Here, we have shown that although the level of neoepitopes is in general lower in PDAC compared with highly immunogenic tumors, a subset of pancreatic cancer cases harbors a significant neoantigens number. As expected, the load of neoantigens was most elevated in cases with microsatellite instability (MSI). The MSI cases were also characterized by higher number of peritumoral lymphocytes and TILs and may represent a minor subset of PDAC that could show response to immune checkpoints blockade. However, we have also observed that PDAC with a higher number of cancerspecific epitopes are characterized by prominent immunosuppressive infiltrate and harbor a microenvironment hostile to T-cell function. Composite analysis with neoantigen burden, immunologic, and stromal features delineated four subtypes of PDAC. Low mutation burden and low levels of immune effector and suppressive cells characterized one of the subtypes. This "cold" subtype harbored KRAS mutations; however, it was underrepresented for many of the canonical PDAC-related genetic events (e.g., SMAD4 or CDKN2A loss). On the basis of the absence of MCT4 and CA9 expression, this subtype was likely predominantly utilizing oxidative phosphorylation, which may correspond to low levels of immunosuppression. Higher stromal volume and presence of collagen-rich stroma could contribute to limited recruitment of immune cells and also result in a paucity of immunosuppressive cells. This subtype could be therefore amenable to approaches activating immune system, such as anticancer vaccines (e.g., MUC1, GVAX) or use of chimeric antigen receptor T-cell therapy with immunomodulating agents to off-set immunosuppressive mechanisms that may emerge with increased immune infiltration posttherapy. The "mutationally cold" subtype also harbored a low number of mutations, however it exhibited low stromal volume and immature stromal type with high-levels of MCT4 indicating a glycolytic and acidic microenvironment. The immune infiltrate in this subtype was dominated by macrophages and could benefit from therapies targeting macrophages, such as CD40 agonists that induce tumoricidal macrophage function and induce T-cell infiltrate (56). The two subsets termed "hot" and "mutationally active" harbored a relatively high mutational burden, higher numbers of TILs and peritumoral lymphocytes, as well as immune checkpoints (CTLA-4 and PD-1) and regulatory T cells but exhibited variable levels of tumor-associated macrophages. Notably, immunosuppressive features of M2 macrophages would ostensibly persist in the face of CTLA4 and PD1 inhibitors. Similarly, a high concentration of lactate could similarly weaken immune responses and lead to therapeutic resistance (57, 58). Therefore, normalizing TME metabolism or bio logic features of tumor stroma and/or combination therapies targeting multiple immunosuppressive mechanism may be required to successfully implement immunotherapy in PDAC. Interestingly, in spite of substantial genomic sequencing efforts, it is not possible to predict tumor/stroma features that are of clear relevance to immune engagement. However, potentially with greater number of cases sequenced it is possible that specific correlations will begin to emerge.

Together, these data suggest that due to the genetic, stromal, and immunologic diversity of PDAC, it will be important to apply immunotherapy in a targeted fashion. Our data also indicate a need for combinatorial approaches (e.g., simultaneously targeting distinct immunosuppressive mechanisms, applying immunotherapy in concert with modifying metabolic reprogramming in tumor microenvironment, activating immune responses via adaptive cell transfer) and provides potential explanations to why single-agent trials with immune checkpoint inhibitors have not been as promising as hoped.

Disclosure of Potential Conflicts of Interest
I.W. Botros and D.M. Thompson hold ownership interest (including patents) in HTG Molecular Diagnostics. V.P. Balachandran reports receiving commercial research grants from Bristol-Myers Squibb. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: E. Knudsen, I.W. Botros, D.M. Thompson, A.K. Wituśkiewicz
Development of methodology: A.K. Wituśkiewicz
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Ngo, I.W. Botros, D.M. Thompson, T.A. Chan, A.K. Wituśkiewicz
Writing, review, and/or revision of the manuscript: E. Knudsen, P. Vail, I.W. Botros, V.P. Balachandran, S.D. Leach, D.M. Thompson, T.A. Chan, A.K. Wituśkiewicz
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E. Knudsen, T.A. Chan
Study supervision: A.K. Wituśkiewicz

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