Cancer Research

Immune-Related Gene Expression Profiling After PD-1 Blockade in Non–Small Cell Lung Carcinoma, Head and Neck Squamous Cell Carcinoma, and Melanoma

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Abstract

Antibody targeting of the immune checkpoint receptor PD1 produces therapeutic activity in a variety of solid tumors, but most patients exhibit partial or complete resistance to treatment for reasons that are unclear. In this study, we evaluated tumor specimens from 65 patients with melanoma, lung nonsquamous, squamous cell lung or head and neck cancers who were treated with the approved PD1-targeting antibodies pembrolizumab or nivolumab. Tumor RNA before anti-PD1 therapy was analyzed on the nCounter system using the PanCancer 730-Immune Panel, and we identified 23 immune-related genes or signatures linked to response and progression-free survival (PFS). In addition, we evaluated intra- and interbiopsy variability of PD1, PD-L1, CD8A, and CD4 mRNAs and their relationship with tumor-infiltrating lymphocytes (TIL) and PD-L1 IHC expression. Among the biomarkers examined, PD1 gene expression along with 12 signatures tracking CD8 and CD4 T-cell activation, natural killer cells, and IFN activation associated significantly with nonprogressive disease and PFS. These associations were independent of sample timing, drug used, or cancer type. TIL correlated moderately (∼0.50) with PD1 and CD8A mRNA levels and weakly (∼0.35) with CD4 and PD-L1. IHC expression of PD-L1 correlated strongly with PD-L1 (0.90), moderately with CD4 and CD8A, and weakly with PD1. Reproducibility of gene expression in intra- and interbiopsy specimens was very high (total SD <3%). Overall, our results support the hypothesis that identification of a preexisting and stable adaptive immune response as defined by mRNA expression pattern is reproducible and sufficient to predict clinical outcome, regardless of the type of cancer or the PD1 therapeutic antibody administered to patients. *Cancer Res; 77(13); 3540–50. ©2017 AACR.*

Introduction

Tumor cells suffer numerous genomic alterations, generating neoantigens that can be identified by the immune system. Although an innate immune response is observed in patients with cancer, this response is usually ineffective to control tumor progression (1, 2). To date, many immune escape mechanisms have been identified (1, 3, 4), including profound local immune suppression, induction of tolerance, dysfunction in T-cell signaling, and evasion of immune destruction by expression of endogenous "immune checkpoints" that normally terminate immune responses after antigen activation. These observations have resulted in the development of various immune approaches for the treatment of cancer, including immune checkpoint pathway inhibitors such as anti-PD1 for the treatment of patients with advanced lung cancer, melanoma, renal cancer, and other tumor types (5–9).

A particular challenge in cancer anti-PD1 immunotherapy is the identification of predictive biomarkers to identify responders from nonresponders and to guide disease-management decisions. Emerging data suggest that patients whose tumors overexpress PD-L1 by IHC have improved clinical outcomes with anti-PD1–directed therapy tumors (10). Although this might be the case of lung cancer, in which PD-L1 IHC does seem predictive, it is not the case of many other cancers, like melanoma or renal cancer, in which results are more controversial. In addition, IHC-based detection of PD-L1 as a predictive biomarker is confounded by multiple issues, many still unresolved today, such as variable

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detection antibodies and cutoffs, tissue preparation, stability of the biomarker in primary versus metastatic biopsies, and staining of tumor versus immune cells (10).

The development of gene expression profiling of tumors has enabled to identify prognostic gene expression signatures and patient selection with targeted therapies. Recently reported studies have evaluated the association of immune-related gene expression in patients with various solid tumors treated with immunotherapy. For example, a genome-wide analysis of serial melanoma biopsies from patients treated with recombinant IL2 revealed a signature predictive of clinical response from pretreatment biopsies (11). More recently, an IFN-inflammatory immune gene expression signature associated with both enhanced overall response (OR) rates and progression-free survival (PFS) in patients with melanoma treated with pembrolizumab, which is subsequently being investigated in other malignancies (12). Other example include an eight-gene signature reflecting preexisting immunity, the T-effector/IFNγ signature, explored in a phase II trial of previously treated non–small cell lung carcinoma (NSCLC; ref. 13). If validated, the implementation of these signatures will require the utilization of robust and reproducible genomic-based platforms.

Here, we evaluated the association of immune-related gene expression profiles in patients with advanced nonsquamous NSCLC (nonsqNSCLC), squamous NSCLC (sqNSCLC), head and neck squamous cell carcinoma (HNSCC), and skin cutaneous melanoma (SKCM). The overall hypothesis is that immune signatures analyzed before anti-PD1 therapy can predict response to PD1 checkpoint blockade independently of cancer type.

Patients and Methods

Patient data

This study included patients with advanced nonsqNSCLC, sqNSCLC, HNSCC, or SKCM, treated at two institutions in Barcelona (Vall d'Hebron Hospital and Hospital Clínic) with anti-PD1 monotherapy in various clinical trials. Patients received pembrolizumab or nivolumab until progression or unacceptable toxicity. Patients with advanced melanoma could have received prior anti-CTLA4 therapy.

Written informed consent was obtained from all patients before enrollment. The hospital Institutional Review Board approved the study in accordance with the principles of Good Clinical Practice, the Declaration of Helsinki, and other applicable local regulations.

Gene expression analysis

A section of the formalin-fixed paraffin-embedded (FFPE) tissue was first examined with hematoxylin and eosin staining to confirm presence of invasive tumor cells and determine the tumor area. For RNA purification (Roche High Pure FFPET RNA Isolation Kit), ≥1–5 10-μm FFPE slides were used for each tumor specimen, and macrodissection was performed, when needed, to avoid normal contamination (14–16). A minimum of approximately 50 ng of total RNA was used to measure the expression of 730 immune-related genes and 40 housekeeping genes using the nCounter platform (NanoString Technologies) and the PanCancer Immune Profiling Panel (17). Data were log base 2–transformed and normalized using housekeeping genes selected using the nSolver 2.6 package. Raw gene expression data has been deposited in Gene Expression Omnibus (GSE93157).

Sample data

All tumor samples were obtained before anti-PD1 therapy. Two different categories of sample-type were considered: sample acquisition before anti-PD1 therapy initiation (≤3 months and no treatment between the biopsy and the beginning of treatment with anti-PD1; named here "baseline") or archival sample (named here "archival"). Thus, an archival sample is a tumor biopsy (either from a primary tumor or a metastatic biopsy) obtained > 3 months before anti-PD1 therapy initiation.

Immune gene signatures

Fifteen independent gene signatures tracking different cell types [e.g., CD8 T cells, NK cells, and dendritic cells (DC)] were evaluated (17–19). A signature that tracks normal mucosa was also included as a control. In addition, we performed an unsupervised analysis using all immune-related genes and all samples, and handpicked four newly developed immune-related signatures that met the following criteria: >15 genes and a correlation coefficient (r) among the genes >0.8. The gene lists of each signature can be found in Supplementary Table S1. To obtain a single score for each signature and sample, median expression of all genes from the signature was calculated.

The Cancer Genome Atlas dataset

All RNASeqv2 samples from nonsqNSCLC, sqNSCLC, HNSCC, and SKCM samples ($n = 2{,}170$) available at the Cancer Genome Atlas (TCGA) portal (https://gdc.cancer.gov/) were downloaded (20). RNA-Seq by expectation maximization (RSEM) values below 3 were treated as missing values, and if more than 50% of expression values were missing for a gene, that particular gene was removed from the data matrix. RSEM values were then log base 2 transformed.

Reproducibility analysis

Expression of PD1, PD-L1, CD8A, and CD4, together with 5 housekeeping genes, was determined across 1,150 FFPE-based samples from 13 cancer types using a custom-made CodeSet (data not shown). The entire expression data was scaled from 0 to 10. To evaluate the variability of PD1, PD-L1, CD8A, and CD4 mRNA expression within the same sample (intrasample variability), an average of 2.6 extractions from a single biopsy/sample (i.e., same tumor cylinder) were profiled in 35 patients, and total SD was calculated. To determine the output variability across different core needle biopsies of the same tumor (intersample variability), 15 biopsies from 5 independent tumors were evaluated, and total SD was calculated.

Tumor-infiltrating lymphocytes and PD-L1 IHC

Percentages of stromal tumor-infiltrating lymphocyte (TIL) were evaluated in hematoxylin and eosin slides from 51 tumor samples according to the 2014 Guidelines developed by the International TILs Working (21). Percentages of PD-L1–expressing tumor cells were assessed in six freshly-cut FFPE lung tumor samples using the commercially available Ventana Sp263 assay.

Statistical analysis

To identify genes differentially expressed across groups, a multiclass significance analysis of microarrays using a FDR <5% was

used. Time from the first day of treatment to progression or last follow-up was defined as PFS. Estimates of PFS were from Kaplan–Meier curves and tests of differences by log-rank test. Multivariable Cox proportional hazards models were built including cancer-type, timing of biopsy, gender, and drug as covariables. Stepwise regression was used to select the most informative variables, which were included in a multiple (linear) regression model. Tumor response was determined at 6 to 8 weeks of starting treatment using modified RECIST 1.1 criteria. Complete response (CR) was defined as CR. OR was defined as partial or CR. Nonprogressive disease (NPD) was defined as response or stable disease (SD) for at least 3 months. Association between gene or signature expression and CR, OR, or NPD rates was also assessed by logistic regression analysis. All statistical tests were two-sided.

Results

Clinical–pathologic characteristics

Tumor samples (50.8% baseline and 49.2% archival) from 65 patients (77% males) with different advanced cancers [NSCLC including both nonsqNSCLC ($n = 22$) and sqNSCLC ($n = 13$); HNSCC ($n = 5$) and SKCM ($n = 25$)] were evaluated in this study (Table 1). All patients had been recruited in various clinical trials evaluating the efficacy of anti-PD1 monotherapy [nivolumab (56.9%) or pembrolizumab (43.1%)]. Most patients (80%) received $\leq$2 prior lines of therapy. In the melanoma group, 7 of 25 patients (28%) had been previously treated with ipilimumab. In 6 of the 7 patients, the archival biopsy was prior to ipilimumab and the biopsy was after ipilimumab in 1 patient. Of the 9 patients with BRAF-mutated melanoma, 3 received a BRAF inhibitor before anti-PD1. In all of them, the biopsy was archival before BRAF-inhibition. In the other groups, no patient received checkpoint inhibitors before anti-PD1 therapy. In the entire population, the OR and NP rates were 30.8% and 55.4%, respectively. Median PFS was 3.47 months [95% confidence interval (CI), 2.8–6.87]. No significant differences in terms of OR and NPD rates, or PFS, were observed across the four cancer types (Supplementary Table S2).

Table 1. Clinical–pathologic characteristics of the combined datasets evaluated in this study

	***N* (%)**
N	65
Age, median (range)	59 (40–83)
Sex	
Male	50 (77%)
Female	15 (23%)
Type of cancer	
Nonsquamous lung carcinoma	22 (34%)
Squamous lung carcinoma	13 (20%)
Squamous head and neck	5 (8%)
Melanoma	25 (39%)
Previous lines	
0	17 (26%)
1	22 (34%)
2	13 (20%)
3	13 (20%)
Melanoma previous immunotherapy	
Ipilimumab	7 (28%)
No immunotherapy	18 (72%)
Biopsy	
Archival	32 (49%)
Baseline	33 (51%)
Drug response	
CR	3 (5%)
PR	17 (26%)
SD	16 (25%)
PD	29 (45%)
ORR	
CR–PR	20 (31%)
SD–PD	45 (69%)
Smoking	
Current smoker	16 (25%)
Former smoker	28 (43%)
Never smoker	21 (32%)
ECOG	
0	20 (31%)
1	43 (66%)
2	2 (3%)
Drug	
Nivolumab	28 (43%)
Pembrolizumab	37 (57%)
PFS, median (95%, CI)	3.47 (2.8–6.87)
Melanoma BRAF status	
BRAF mutated	9 (36%)
Previous BRAF inhibitor	*3 (12%)*
Nonprevious BRAF inhibitor	*6 (24%)*
BRAF wild-type	16 (64%)
Lung cancer EGFR status	
EGFR mutated	1 (3%)[a]
EGFR wild-type	30 (75%)
NA	9 (23%)
Lung cancer ALK status	
ALK rearranged	0 (0%)
ALK not rearranged	30 (75%)
NA	10 (25%)

Abbreviations: CR, complete response; PR, partial response; SD, stable disease; PD, progression disease; ORR, overall response rate; PFS, progression-free survival; ALK, anaplastic lymphoma kinase; NA, not applicable.
[a]Previously treated with EGFR TKI.

Immune cell-type expression across cancer types

To identify immune cell-type expression across the different cancer types, we performed an unsupervised analysis of 730 immune-related genes and 65 samples (Fig. 1). The gene clustering revealed that most genes ($n = 536$, 73.4%) were highly correlated (correlation coefficient >0.50). The sample clustering revealed two clear groups of samples (groups 1 and 2). No clear associations were observed between the two clusters of samples and response to therapy (i.e., PD, SD, and OR), timing of biopsy, or sex. Interestingly, a significant association ($P < 0.001$, Fisher exact test) was observed regarding the type of cancer, where most sqNSCLC and SKCM samples clustered together in group 2 and showed an overall pattern of lower expression of immune-related genes than nonsqNSCLC samples. Of note, a significant association ($P = 0.011$, Fisher exact test) was found regarding the two groups and the drug used as most nonsqNSCLC patients were treated with nivolumab.

From the hierarchical clustering (Fig. 1), we handpicked four gene clusters (i.e., cluster 1, cluster 2, cluster 3, and cluster 4). Cluster 1 ($n = 68$ genes) tracks antigen presentation through T-cell activation. The signature is composed of genes that form the TCR complex (e.g., CD3D, CD3E, CD3G, CD247, and ZAP70), costimulate T cells (i.e., CD2, CD28, and ICOS), and participate in the differentiation of naïve T cells into CD4 Th1 cells (e.g., IL12RB1, CXCR3, and STAT4). Cluster 2 ($n = 20$) tracks granzyme A–mediated apoptosis pathway (i.e., granzyme A and B and perforin 1), checkpoint inhibitors (i.e., PD1 and LAG3), and T-cell receptor signaling (e.g., CD8A, CD8B, and IFNγ). Cluster 3 ($n = 44$) tracks cell-adhesion molecules (e.g., CD4, CD86, and integrin β2), toll-like receptors (i.e., toll-like

receptor 1, 4, 7, and 8), and immune checkpoint inhibitor PD-L2. Finally, Cluster 4 ($n = 17$) tracks IFN activation (i.e., IFN A7) and T-cell CD4 Th2 activation (i.e., IL13).

Cancer-type immune-specific profiles

To identify cancer-type immune-specific profiles, we first retrieved RNA-Seq data from TCGA project for a total of 2,170 tumor samples representing HNSCC ($n = 566$), sqNSCLC ($n = 554$), nonsqNSCLC ($n = 576$), and SKCM ($n = 474$). SqNSCLC and HNSCC samples were combined into a single group because their gene expression patterns are largely undistinguishable as previously reported by the TCGA pan-cancer group (22). Of note, >90% of samples from the TCGA project are primary tumors.

In the TCGA dataset, the expression of all immune-related genes, or the genes directly associated with immune cell types, was found to discriminate (FDR < 5%) the three cancer groups (Fig. 2A). Interestingly, very similar patterns of immune-related expression were observed in our nCounter-based dataset, where nonsqNSCLC samples show higher expression of immune-related genes, or immune cell-type genes, than the other cancer groups, especially SKCM samples (Fig. 2A). For example, PD-L1 gene was found less expressed in melanoma samples than the other cancer groups (Fig. 2B). The expression of the 23 genes and signatures across the three cancer groups in the nCounter-based dataset are shown in Supplementary Fig. S1. Of note, virtually all our nCounter-based data originates from core biopsies from metastatic tissues.

Using all genes evaluated on the nCounter platform, we derived a gene expression–based predictor in the TCGA dataset that identifies each of the three cancer groups, and then applied this predictor onto our nCounter-based dataset. The predictor identified the correct cancer group in 86.2% (56/65) of the cases (Fig. 2C). When the subset of genes focusing only on immune cell-types was evaluated, the predictor identified the correct cancer group in 80% (52/65) of the cases (Fig. 2C).

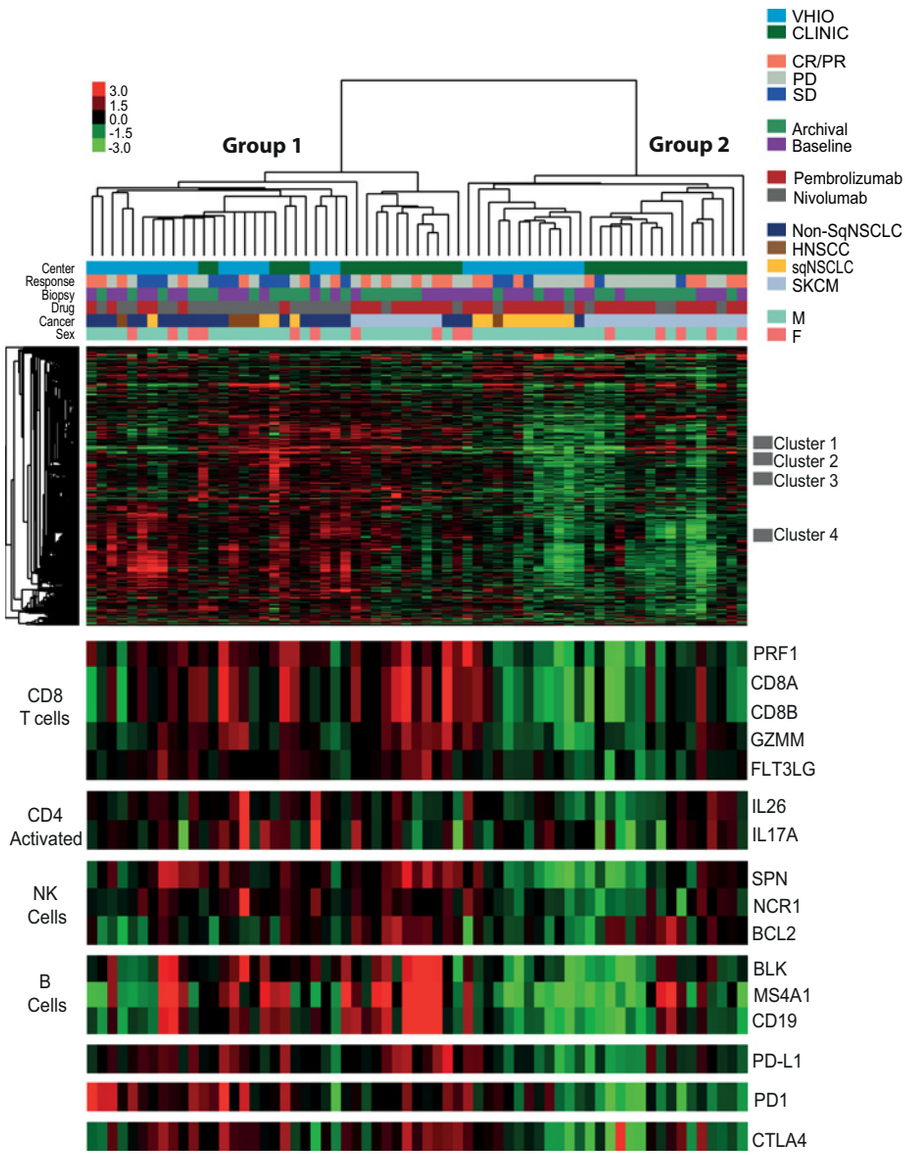

Figure 1.
Immune-related gene expression across cancer types. Hierarchical clustering of 65 tumors using 730 immune-related genes. Genes were median centered. Each colored square represents the relative mean transcript abundance (in $\log_2$ space) for each sample, with highest expression shown in red, median expression in black, and lowest expression in green. Type of biopsy, drug, cancer type, and sex are identified below the array tree. Four handpicked gene clusters are shown on the right of the heatmap. Below the heatmap, the expression of selected gene signatures or genes is shown.

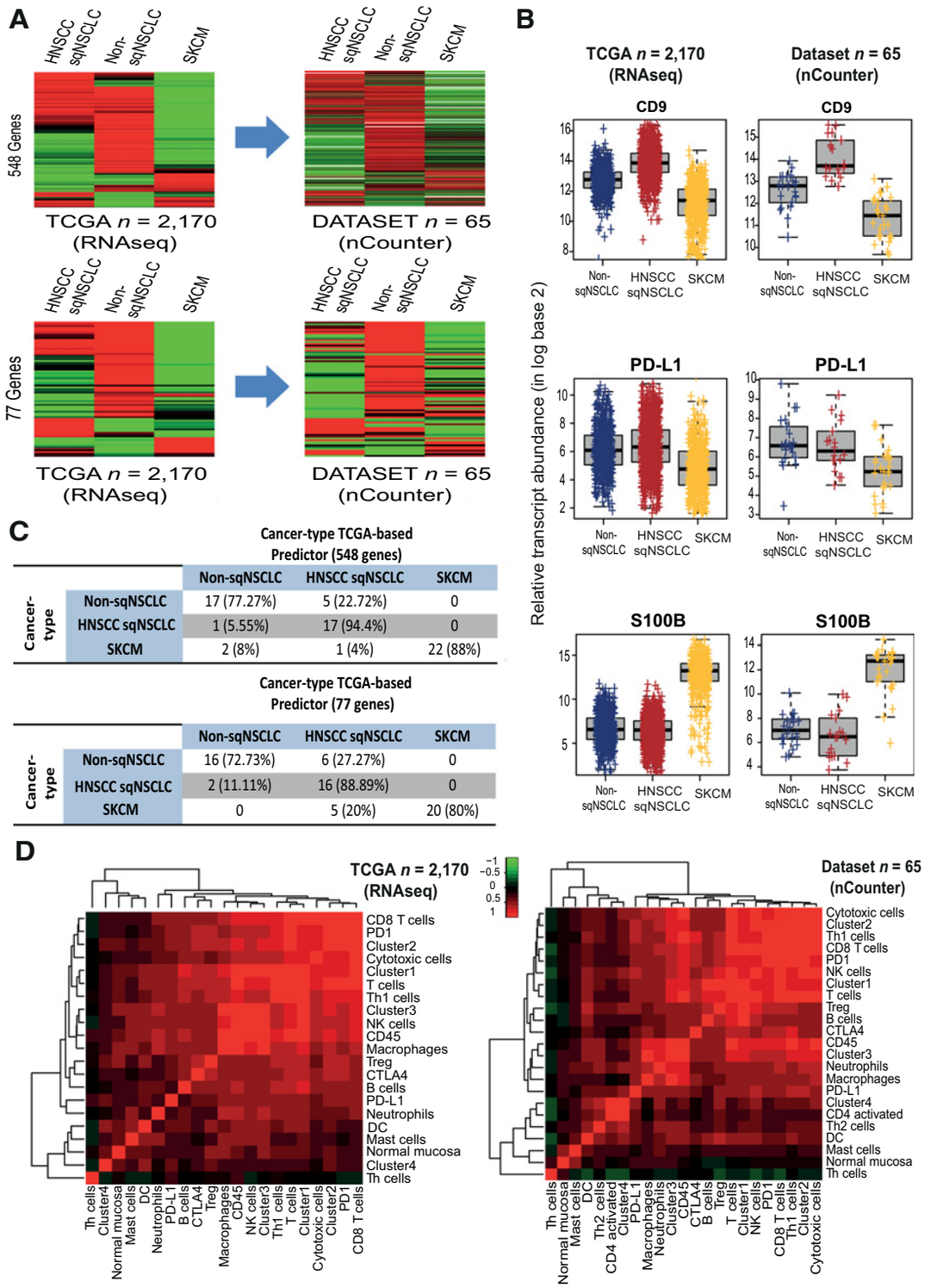

Figure 2.

Cancer-type immune-specific profiles. **A,** Immune-related profiles, and immune cell–type–specific profiles, in the TCGA dataset compared with the nCounter-based dataset. SqNSCLC and HNSCC were combined into a single group based on the TCGA PanCancer results (22). **B,** Expression of selected genes across the three cancer groups in the TCGA (left) and nCounter-based (right) datasets. **C,** Prediction of cancer group in the nCounter-based dataset using two different TCGA gene expression–based predictors. Top, predictions using all immune-related genes. Bottom, predictions using all cell-type–specific genes. Both gene lists can be found in the Supplementary Data. **D,** Correlation among immune cell–type signatures and selected genes (PD1, PDL1, and CTLA4) in the TCGA dataset (left) and nCounter-based dataset (right).

Correlation among PD1, PD-L1, and immune cell–type signatures

To better understand the association between the various signatures being analyzed, we performed a hierarchical clustering of pairwise correlations between the 20 signatures, and PD1, PD-L1, and CTLA4 genes, in the combined cohort of 65 patients (Fig. 2D). Among the different signatures, six (i.e., cytotoxic cells, CD8 T cells, T cells, NK cells, Th1 cells, cluster 1, and cluster 2) tracking cytotoxic T-cell infiltration, as well as PD1 gene, showed high correlation among them (correlation coefficient > 0.77). Similar results were obtained using the TCGA RNA-seq data (Fig. 2D).

Immune-related gene expression and therapy response/benefit

To identify the immune-related biological processes associated with anti-PD1 therapy response/benefit, we first evaluated the association between the expression of each individual gene, or signature, and type of response/benefit (i.e., ORR and NPD) after adjustment for four clinical–pathological variables (i.e., sex, type of biopsy, cancer type, type of drug). On one hand, CD8 T cells and PD1 showed a clear tendency for being associated with ORR but did not reach statistical significance (Fig. 3A). On the other hand, 14 signatures (cytotoxic cells, NK cells, Th1 cells, cluster 2, CD8 T cells, cluster 1, T cells, cluster 4, cluster 3, CD45, CD4-activated, dendritic cells, neutrophils, and Treg cells), together with PD-L1, PD1, and CTLA4 were significantly associated with NPD (Fig. 3B). Of note, no other clinical–pathologic variable was found clearly associated with NPR, except for sex [male vs. female; odds ratio = 4.89; 95% confidence interval (CI), 1.44–19.76; $P = 0.015$].

Immune-related gene expression and PFS

To identify the immune-related biological processes associated with anti-PD1 therapy response/benefit, we evaluated the association between the expression of each individual gene, or signature, and PFS (Fig. 4A). Among the different biomarkers, 11 signatures (NK cells, cluster 4, CD8 T cells, cluster 2, cluster 1, Th1 cells, T cells, CD4 activated, cytotoxic cells, DC, and Treg cells), together with PD1 and PD-L1 genes were found significantly associated with PFS after adjustment for four clinical–pathologic variables (i.e., sex, type of biopsy, cancer type, type of drug). Sex was the only clinical–pathologic variable found associated with PFS [male vs. female; hazard ratio (HR) = 0.48; 95% CI, 0.25–0.92; $P = 0.02$] in univariate analysis.

Finally, we explored the actual survival outcomes of the patients based on the expression of the six most significant signatures or genes (Fig. 4B; Supplementary Fig. S2). To draw cutoffs, we used tertiles and defined low expressers as those in the low tertile group and high expressers as those in the high tertile groups. All signatures or genes, except PD1, showed a significant association with PFS. For example, the median PFS of patients with low and high NK-cell expression was 2.57 and 6.87 months (HR = 0.39; 95% CI, 0.185–0.815). Moreover, the percentage of patients with low and high NK-cell expression who were found progression-free at 12 and 24 months were 9.1% versus 23.8% and 9.1% versus 14.28%, respectively.

PD1, PD-L1, CD8A, and CD4 mRNA output variability

To evaluate the variability of PD1, PD-L1, CD8A, and CD4 mRNA expression within the same sample, multiple extractions from a single biopsy/sample (i.e., same tumor cylinder) were profiled. A total of three extractions from three independent core needle biopsies (total of 35 patients) were tested (average of 2.6 extractions per core needle biopsy). The SD for PD1, PD-L1, CD8A, and CD4 mRNA was 0.136, 0.05, 0.046, and 0.03 units, respectively, in a scale of 0–10 (intrabiopsy variability).

To determine the output variability across different core needle biopsies of the same tumor, a total of 15 biopsies from five independent tumors (average of 2.5 biopsies per tumor) were evaluated. The SD for PD1, PD-L1, CD8A, and CD4 mRNA was 0.239, 0.04, 0.12, and 0.07 units, respectively, in a scale of 0 to 10 (interbiopsy variability). Overall, these results support the high reproducibility of the nCounter platform in evaluating immune-related expression profiles.

Correlation TILs and PD-L1 IHC with immune genes

We first investigated the correlation of stromal TILs with PD1, PD-L1, CD8A, and CD4 mRNA in 51 evaluable samples. Among the four biomarkers evaluated, PD1 and CD8A were found moderately correlated [correlation coefficients (r) of 0.54 and 0.53], and CD4 and PD-L1 were found weakly correlated ($r = 0.37$ and 0.35; Supplementary Fig. S3). Second, tumor PD-L1 IHC was found strongly correlated ($r = 0.90$) with PD-L1, moderately correlated (0.53 and 0.42) with CD4 and CD8A, and weakly correlated ($r = 0.25$) with PD1 (Supplementary Fig. S4).

Discussion

In the last few years, anti-PD1/PD-L1 drugs have become a new paradigm in oncology (23, 24). Nivolumab and pembrolizumab, both monoclonal IgG4 antibodies against PD1, have demonstrated significant response rates in several clinical trials involving patients with advanced SKCM, lung cancer and other solid tumors (5–9). However, not all patients benefit and those who benefit do not benefit to the same extend. Thus, there is a need to better understand the biology behind the activity of these drugs and identify predictive biomarkers of response.

The development of tools for selecting patients that are likely to benefit from these therapies has been investigated but still remains unclear. At present time, PD-L1 expression by IHC remains the only potential biomarker, but important inherent limitations exists (10). In this way, the Blueprint Proposal (25) has been developed by four pharmaceutical companies and two diagnostic companies involved in the immune-oncology field. The proposed study will help build an evidence base for PD1/PD-L1 companion diagnostic characterization for non–small cell lung cancer aiming to get consensus regarding PD1/PD-L1 as a biomarker in a similar manner as has been done for HER2 or hormone receptor testing in breast cancer (26, 27).

Lessons learned over the years regarding the implementation of IHC-based biomarker guidelines reveal the existence of important discordance rates in the daily clinical setting whether the same antibody, or two different antibodies, are used (28, 29). This is observed for biomarkers that are used as a binary score (i.e., positive or negative). Moreover, even higher discordance rates have been observed with IHC-based biomarkers such as Ki67 when there is a need to quantify (i.e., percentages of positive tumor cells) and draw specific cutoffs (30). Thus, it is likely that PD1/PD-L1 IHC determinations will suffer from similar issues. In this scenario, implementation of robust and reproducible assays under genomic platforms such as the nCounter is needed. For example, a genomic assay that measures 50 genes (i.e., PAM50) using the nCounter platform has already been implemented in the

clinical setting with high reproducibility within and across labs (31). Thus, studies that evaluate the analytic validation of immune-related genes or signatures using robust genomic platforms seem warranted. In this direction, our variability analyses with PD1, PD-L1, CD8A, and CD4 mRNA, together with reported data by NanoString of the analytic performance of an immune

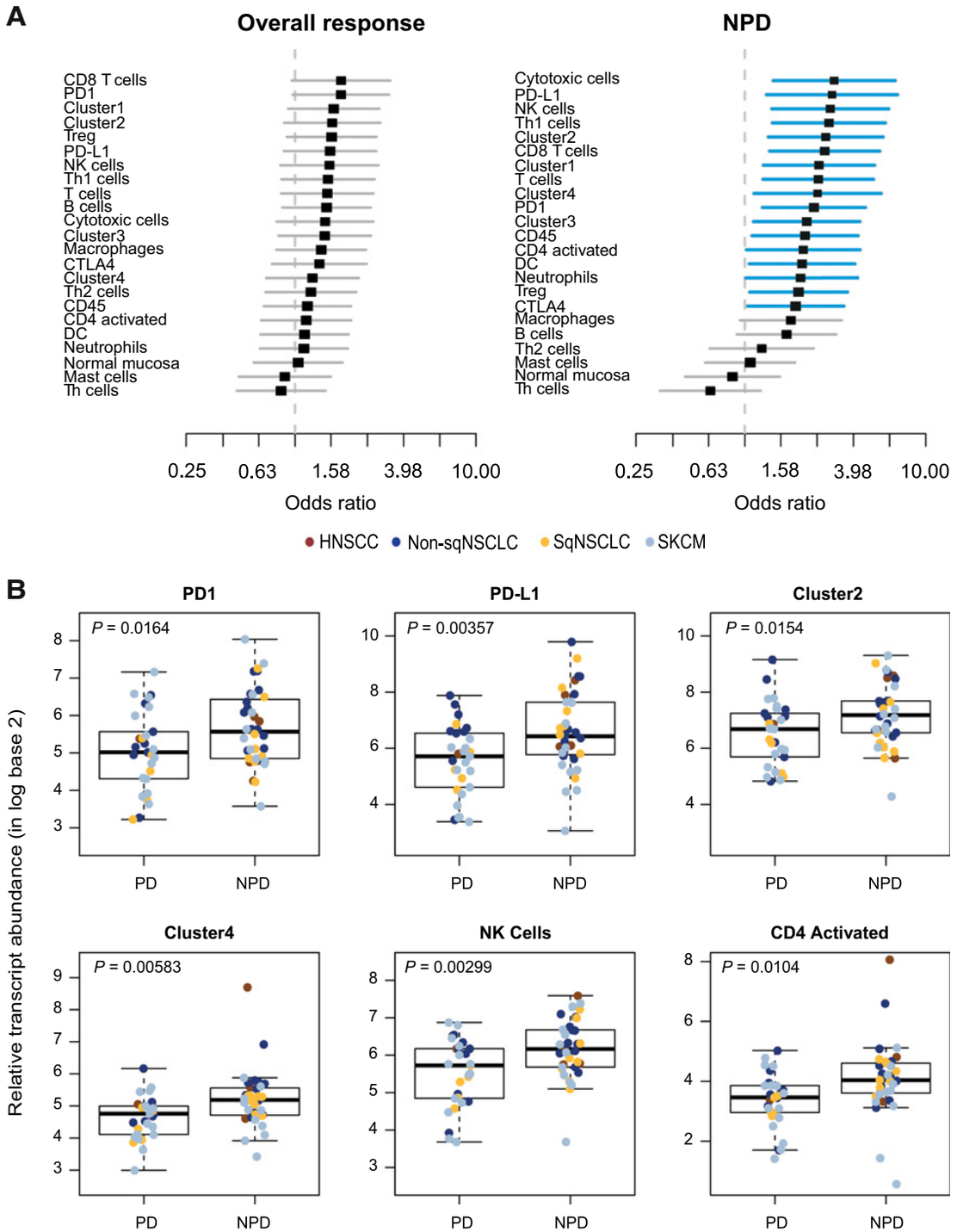

Figure 3.

Immune-related gene expression and therapy response/benefit. **A,** Odds ratios of various genes or gene signatures (as a continuous variable) for odds ratio (left) and NPD (right), after adjusting for clinical–pathologic variables. Each signature was standardized to have a mean of 0 and a SD of 1. The size of the square is inversely proportional to the SE. Horizontal bars represent the 95% CIs of odds ratios. Statistically significant variables are shown in blue. Each gene signature was evaluated individually and rank ordered on the basis of the estimated odds ratio. **B,** Box-and O whisker plots for expression of selected genes or gene signatures across patients that showed PD and NPD. Each colored circle represents a cancer type.

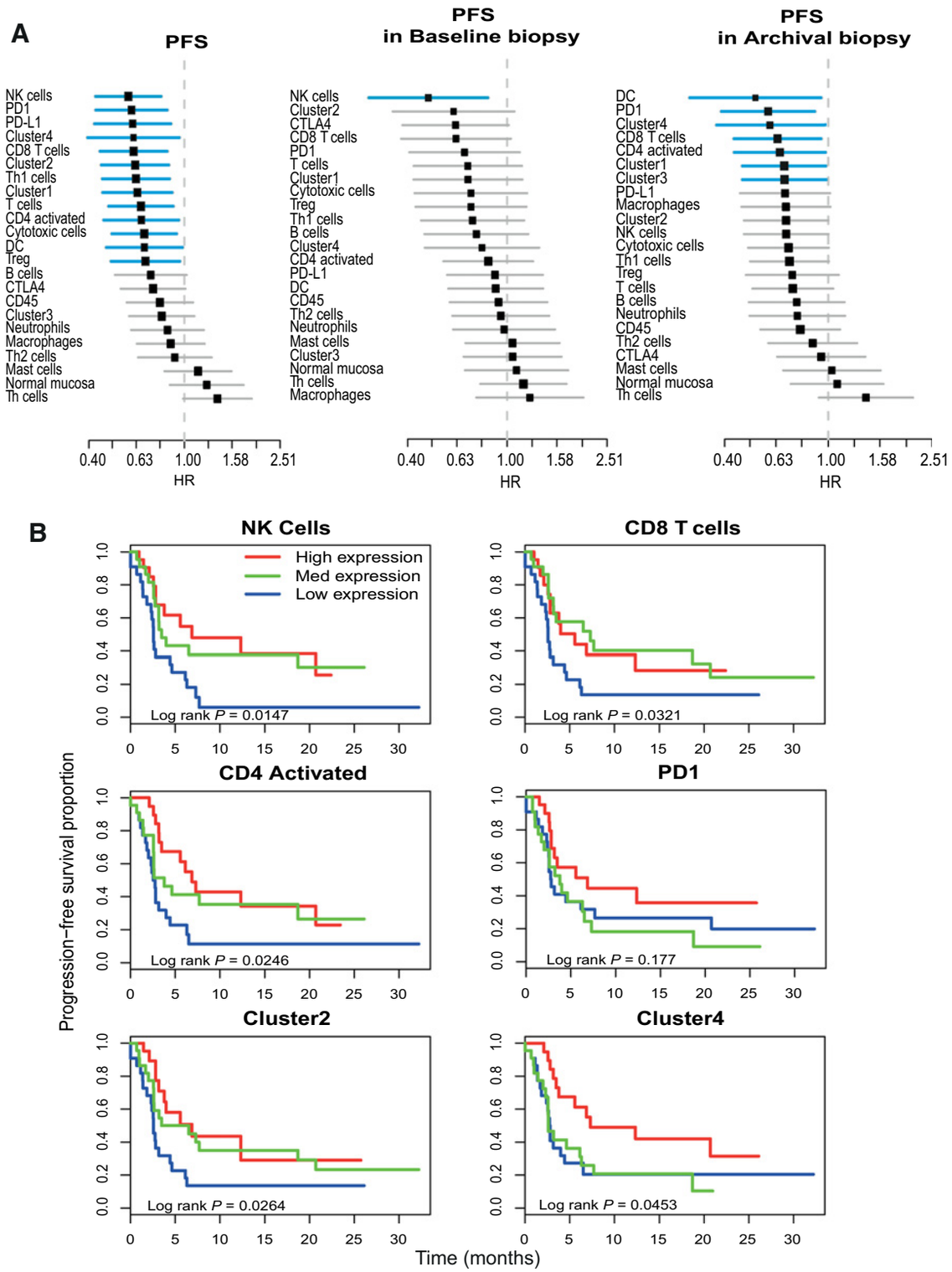

Figure 4.

Immune-related gene expression and PFS. **A,** PFS HRs of various genes or gene signatures (as a continuous variable) in all patients (left), patients with a baseline biopsy (middle), and with an archival biopsy (right). Each signature was standardized to have a mean of 0 and a SD of 1. The size of the square is inversely proportional to the SE. Horizontal bars represent the 95% CIs of HRs. Statistically significant variables are shown in blue. Each gene signature has been evaluated individually. Each gene or signature was rank ordered on the basis of estimated HR. **B,** Kaplan–Meier survival analysis based on selected genes or gene signatures. Each gene or signature was divided into tertiles based on their expression.

gene signature (32), support the high reproducibility of the nCounter platform for measuring immune-related biomarkers.

Two previous articles have evaluated the association of the transcriptome and response to anti-PD1 monotherapy (33, 34). In the first one, Hugo and colleagues (33) compared the expression of more than 25,000 genes using RNAseq between 15 responding (i.e., defined as NPD) and 13 nonresponding (i.e., defined as progressive disease) patients with advanced melanoma treated with pembrolizumab or nivolumab. The authors identified 693 genes differentially expressed, mostly upregulated in nonresponding tumors, between the two groups. Interestingly, mesenchymal and inflammatory tumor phenotypes were found associated with innate anti-PD1 resistance. However, genes with putative roles in modulating immune checkpoint sensitivity, such as PD-L1, CD8A/B, IFNγ, or multiple IFN signatures, were not found associated with NPD. In the second study, Ascierto and colleagues (34) compared the expression of 60 immune-related genes using multiplex qRT-PCR in 12 patients (4 responders and 8 nonresponders) with PD-L1–positive advanced renal cell carcinoma treated with nivolumab. No gene was found differentially expressed between the 2 groups. In a subsequent analysis evaluating the whole transcriptome with RNA-seq, the expression of metabolic-related genes, and not genes involved in immune checkpoint sensitivity, were found associated with anti-PD1 resistance. Although we do not have a clear explanation of why these results are different from ours, the differences in the number of samples, the cancer-type (i.e., renal carcinoma) and the transcriptomic approach may not be comparable.

On the contrary, results from two recently reported studies evaluating the association of immune-related gene expression in patients with various solid tumors treated with anti-PD1/PD-L1 are consistent with ours. In the first one, Ayers and colleagues developed a predictive "IFNγ" gene signature using the nCounter platform in 19 patients with advanced SKCM treated with pembrolizumab (35). Then they tested the predictive ability of this signature, and three additional signatures, in patients with SKCM ($n = 62$), HNSCC ($n = 33$), and gastric cancer ($n = 33$) treated with pembrolizumab in KEYNOTE-001 and KEYNOTE-012 trials. Overall, they observed that tumors lacking an immune phenotype, as suggested by low scores of the signatures, did not respond to anti-PD1 therapy (35). In the second study, Fehrenbacher and colleagues (13) evaluated 224 NSCLC pretreatment samples from a phase II trial where patients were randomized to docetaxel or atezolizumab, an anti-PD-L1 drug. The authors observed that patients with high T-effector-IFNγ-associated gene expression, measured using the Nimblegen platform, had improved overall survival with atezolizumab. These studies support our findings that a similar immune biology related to T cell and IFN activation predicts anti-PD1/PD-L1 response or benefit across multiple cancer types and that the benefit from checkpoint inhibition is pronounced in tumors with preexisting immunity. Further supporting this hypothesis is a recent study using flow cytometry on freshly isolated metastatic melanoma samples from two cohorts of 20 patients, revealing that increasing fractions of PD1-high/CTL–associated protein four high (PD1hiCTLA-4hi) cells within the tumor-infiltrating $CD8^+$ T-cell subset strongly correlates with response and PFS following anti-PD1 monotherapy (36).

The gene signatures identified here (e.g., cluster 2 and CD8 T cells), and in previous reports (13, 35), as being associated with anti-PD1 therapy response or survival are composed of genes previously identified as the Immunologic Constant of Rejection (ICR; e.g., CXCR3, CCR5 ligand genes, and IFNγ-signaling transcripts; 18, 37). In this context, predictive, prognostic, and mechanistic immune signatures overlap, and a continuum of intratumor immune reactions exists. Indeed, ICR-related genes have been constantly found associated with increased survival across cancer types (19, 38) and responsiveness to immunotherapeutic approaches including anti-CTLA4 (39), adoptive therapy/IL2 (40, 41), and vaccination (41).

Beyond PD-L1 IHC and gene expression signatures, other potential genomic biomarkers of response or benefit to immune checkpoint inhibitors are emerging (42, 43). For example, there is a link between the number of mutations (the so-called mutational load), the formation of neoantigens, and immune response (44). Indeed, cancer types with the highest response to anti-PD1 (i.e., SKCM and lung cancer) to date are the ones to have a high mutational load. For example, McGranahan and colleagues (45) found that a high burden of clonal tumor neoantigens correlated with improved patient survival, an increased presence of TILs, and a durable response to anti-PD1 and anti-CTLA4. Moreover, Rizvi and colleagues (46) studied the tumors of patients with NSCLC undergoing anti-PD1 therapy. Across two independent cohorts, higher nonsynonymous mutation burden in tumors was associated with improved objective response, durable clinical benefit, and PFS. However, whether this approach predicts benefit in other cancer types beyond NSCLC still needs to be explored. In addition, further studies are needed to explore the analytic validity of exome sequencing and neoantigen identification.

An interesting observation in our study was that very similar patterns of single and global immune-related gene expression profiles existed between our samples and the TCGA tumor samples. Indeed, SKCM, nonsqNSCLC, and sqNSCLC/HNSCC TCGA samples are largely (>90%) from patients with nonmetastatic disease and were obtained from surgical specimens. On the contrary, our samples are from patients with metastatic disease, many of them pretreated with chemotherapy, and obtained from core-needle biopsies. These data suggest that immune activation seems to occur early on in the disease and seems quite stable throughout tumor progression (as measured by gene expression). This hypothesis is supported by our observations that the type of tissue (baseline vs. archival) did not affect substantially the association of our signatures with anti-PD1 response. Moreover, we observed that each cancer type has a unique immune-related or immune cell-type–specific gene expression profile, suggesting that different mechanisms of immune activation or suppression might be occurring and might be cancer-type specific to some extent.

There are several caveats to our study. First, this is an exploratory and retrospective study with a limited sample size; therefore, we did not attempt to find the best biomarker. Thus, prospective and randomized studies will be needed to define and validate the predictive ability of each gene or signature and the best cutoff based on performance and clinical utility. Second, we did not evaluate overall survival since the number of events (i.e., deaths) was insufficient at the time of analysis. Third, the number of HNSCC samples analyzed is low and thus no major conclusions can be drawn in this group. However, as previously discussed, there is already clinical evidence of the predictive value of immune-related gene expression in patients with HNSCC treated with pembrolizumab (35). Fourth, we did not have on-treatment samples to evaluate whether these are more valuable than pretreatment samples. In this direction, Chen and colleagues (47)

analyses suggested that immune profiling identified in early on-treatment biopsies following anti-PD1 blockade is more predictive of benefit compared with pretreatment tumor samples. Finally, we only had 6 samples to compare tumor PD-L1 IHC expression with immune expression. However, the six samples represented a broad range of IHC staining and were able to observe a high correlation with PD-L1 mRNA.

In summary, our results reveal that although each cancer type might have unique immune expression profiles, various genes, including PD1, or signatures, mostly targeting CD8 and CD4 T-cell and IFN activation, are associated with NPD and better progression-free survival independently of cancer type, timing of the biopsy or anti-PD1 drug. The results are consistent with the hypothesis that identification of a preexisting and stable adaptive immune response predicts clinical outcome. Moreover, we show that the nCounter platform offers high intra- and interbiopsy reproducibility. Further clinical validation of these immune-related gene expression profiles seems warranted.

Disclosure of Potential Conflicts of Interest

A. Prat is a consultant/advisory board member for Nanostring Technologies. E. Felip is a consultant/advisory board member for Eli Lilly, Pfizer, Roche, Boehringer Ingelheim, Astra Zeneca, BMS, MSD, and Novartis. No potential conflicts of interest were disclosed by the other authors.

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References

1. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646–74.
2. Fridman WH, Pages F, Sautes-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. Nat Rev Cancer 2012; 12:298–306.
3. Gajewski TF, Schreiber H, Fu YX. Innate and adaptive immune cells in the tumor microenvironment. Nat Immunol 2013;14:1014–22.
4. Medler TR, Cotechini T, Coussens LM. Immune response to cancer therapy: mounting an effective antitumor response and mechanisms of resistance. Trends Cancer 2015;1:66–75.
5. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer 2012;12:252–64.
6. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med 2012;366:2443–54.
7. Borghaei H, Paz-Ares L, Horn L, Spigel DR, Steins M, Ready NE, et al. Nivolumab versus docetaxel in advanced nonsquamous non-small-cell lung cancer. N Engl J Med 2015;373:1627–39.
8. Robert C, Schachter J, Long GV, Arance A, Grob JJ, Mortier L, et al. Pembrolizumab versus ipilimumab in advanced melanoma. N Engl J Med 2015;372:2521–32.
9. Motzer RJ, Escudier B, McDermott DF, George S, Hammers HJ, Srinivas S, et al. Nivolumab versus everolimus in advanced renal-cell carcinoma. N Engl J Med 2015;373:1803–13.
10. Patel SP, Kurzrock R. PD-L1 expression as a predictive biomarker in cancer immunotherapy. Mol Cancer Ther 2015;14:847–56.
11. Weiss GR, Grosh WW, Chianese-Bullock KA, Zhao Y, Liu H, Slingluff CL Jr, et al. Molecular insights on the peripheral and intratumoral effects of systemic high-dose rIL-2 (aldesleukin) administration for the treatment of metastatic melanoma. Clin Cancer Res 2011;17:7440–50.
12. Ribas A, Robert C, Hodi FS, Wolchok JD, Joshua AM, Hwu W-J, et al. Association of response to programmed death receptor 1 (PD-1) blockade with pembrolizumab (MK-3475) with an interferon-inflammatory immune gene signature. J Clin Oncol 33, 2015 (suppl; abstr 3001).
13. Fehrenbacher L, Spira A, Ballinger M, Kowanetz M, Vansteenkiste J, Mazieres J, et al. Atezolizumab versus docetaxel for patients with previously treated non-small-cell lung cancer (POPLAR): a multicentre, open-label, phase 2 randomised controlled trial. Lancet 2016;387:1837–46.
14. Vidal M, Peg V, Galván P, Tres A, Cortés J, Ramón y Cajal S, et al. Gene expression-based classifications of fibroadenomas and phyllodes tumours of the breast. Mol Oncol 2015;9:1081–90.
15. Prat A, Galván P, Jimenez B, Buckingham W, Jeiranian HA, Schaper C, et al. Prediction of response to neoadjuvant chemotherapy using core needle biopsy samples with the prosigna assay. Clin Cancer Res 2016;22:560–6.
16. Prat A, Cheang MC, Galván P, Nuciforo P, Paré L, Adamo B, et al. Prognostic value of intrinsic subtypes in hormone receptor–positive metastatic breast cancer treated with letrozole with or without lapatinib. JAMA Oncol 2016;2:1287–94.
17. Cesano A. nCounter((R)) PanCancer Immune Profiling Panel (NanoString Technologies, Inc., Seattle, WA). J Immunother Cancer 2015;3:42.
18. Galon J, Angell HK, Bedognetti D, Marincola FM. The continuum of cancer immunosurveillance: prognostic, predictive, and mechanistic signatures. Immunity 2013;39:11–26.
19. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pagès C, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science 2006;313:1960–4.
20. Wang K, Singh D, Zeng Z, Coleman SJ, Huang Y, Savich GL, et al. MapSplice: accurate mapping of RNA-seq reads for splice junction discovery. Nucleic Acids Res 2010;38:e178.
21. Salgado R, Denkert C, Demaria S, Sirtaine N, Klauschen F, Pruneri G, et al. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. Ann Oncol 2015;26:259–71.
22. Hoadley KA, Yau C, Wolf DM, Cherniack AD, Tamborero D, Ng S, et al. Multiplatform analysis of 12 cancer types reveals molecular classification within and across tissues of origin. Cell 2014;158:929–44.
23. Ribas A. Releasing the brakes on cancer immunotherapy. N Engl J Med 2015;373:1490–2.
24. Ribas A. Tumor immunotherapy directed at PD-1. N Engl J Med 2012;366:2517–9.
25. Averbuch S, Emancipator K, McCaffery I, McElhinny A, Stanforth D, Walker J, et al. A blueprint proposal for companion diagnostic comparability. Working group proposal presented at: FDA-AACR-ASCO Public Workshop "Complexities in Personalized Medicine: Harmonizing Companion Diagnostics Across a Class of Targeted Therapies. Available from: https://www.fda.gov/downloads/medicaldevices/newsevents/workshopsconferences/ucm439440.pdf.

26. Hammond ME, Hayes DF, Dowsett M, Allred DC, Hagerty KL, Badve S, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer (unabridged version). Arch Pathol Lab Med 2010;134:e48–72.
27. Wolff AC, Hammond MEH, Hicks DG, Dowsett M, McShane LM, Allison KH, et al. Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists Clinical Practice Guideline Update. J of Clin Oncol 2013;31:3997–4013.
28. McCullough AE, Dell'orto P, Reinholz MM, Gelber RD, Dueck AC, Russo L, et al. Central pathology laboratory review of HER2 and ER in early breast cancer: an ALTTO trial [BIG 2-06/NCCTG N063D (Alliance)] ring study. Breast Cancer Res Treat 2014;143:485–92.
29. Perez EA, Suman VJ, Davidson NE, Martino S, Kaufman PA, Lingle WL, et al. HER2 testing by local, central, and reference laboratories in specimens from the North Central Cancer Treatment Group N9831 intergroup adjuvant trial. J Clin Oncol 2006;24:3032–8.
30. Polley M-YC, Leung SCY, McShane LM, Gao D, Hugh JC, Mastropasqua MG, et al. An International Ki67 Reproducibility Study. J Natl Cancer Inst 2013;105:1897–906.
31. Nielsen T, Wallden B, Schaper C, Ferree S, Liu S, Gao D, et al. Analytical validation of the PAM50-based Prosigna Breast Cancer Prognostic Gene Signature Assay and nCounter Analysis System using formalin-fixed paraffin-embedded breast tumor specimens. BMC Cancer 2014;14:177.
32. Wallden B, Pekker I, Popa S, Dowidar N, Sullivan A, Hood T, et al. Development and analytical performance of a molecular diagnostic for anti-PD1 response on the nCounter Dx Analysis System. J Clin Oncol 34, 2016(suppl; abstr 3034).
33. Hugo W, Zaretsky JM, Sun L, Song C, Moreno BH, Hu-Lieskovan S, et al. Genomic and transcriptomic features of response to anti-PD-1 therapy in metastatic melanoma. Cell 2016;165:35–44.
34. Ascierto ML, McMiller TL, Berger AE, Danilova L, Anders RA, Netto GJ, et al. The intratumoral balance between metabolic and immunologic gene expression is associated with anti-PD-1 response in patients with renal cell carcinoma. Cancer Immunol Res 2016;4:726–33.
35. Ayers M, Lunceford J, Nebozhyn M, Murphy E, Loboda A, Albright A, et al. Relationship between immune gene signatures and clinical response to PD-1 blockade with pembrolizumab (MK-3475) in patients with advanced solid tumors. J Immunother Cancer 2015;3:1.
36. Daud AI, Loo K, Pauli ML, Sanchez-Rodriguez R, Sandoval PM, Taravati K, et al. Tumor immune profiling predicts response to anti-PD-1 therapy in human melanoma. J Clin Invest 2016;126:3447–52.
37. Wang E, Bedognetti D, Marincola FM. Prediction of response to anticancer immunotherapy using gene signatures. J Clin Oncol 2013;31:2369–71.
38. Desmedt C, Haibe-Kains B, Wirapati P, Buyse M, Larsimont D, Bontempi G, et al. Biological processes associated with breast cancer clinical outcome depend on the molecular subtypes. Clin Cancer Res 2008; 14:5158–65.
39. Ji RR, Chasalow SD, Wang L, Hamid O, Schmidt H, Cogswell J, et al. An immune-active tumor microenvironment favors clinical response to ipilimumab. Cancer Immunol Immunother 2012;61:1019–31.
40. Bedognetti D, Spivey TL, Zhao Y, Uccellini L, Tomei S, Dudley ME, et al. CXCR3/CCR5 pathways in metastatic melanoma patients treated with adoptive therapy and interleukin-2. Br J Cancer 2013;109:2412–23.
41. Ulloa-Montoya F, Louahed J, Dizier B, Gruselle O, Spiessens B, Lehmann FF, et al. Predictive gene signature in MAGE-A3 antigen-specific cancer immunotherapy. J Clin Oncol 2013;31:2388–95.
42. Manson G, Norwood J, Marabelle A, Kohrt H, Houot R. Biomarkers associated with checkpoint inhibitors. Ann Oncol 2016;27:1199–206.
43. Gubin MM, Zhang X, Schuster H, Caron E, Ward JP, Noguchi T, et al. Checkpoint blockade cancer immunotherapy targets tumour-specific mutant antigens. Nature 2014;515:577–81.
44. Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SA, Behjati S, Biankin AV, et al. Signatures of mutational processes in human cancer. Nature 2013; 500:415–21.
45. McGranahan N, Furness AJ, Rosenthal R, Ramskov S, Lyngaa R, Saini SK, et al. Clonal neoantigens elicit T cell immunoreactivity and sensitivity to immune checkpoint blockade. Science 2016;351:1463–9.
46. Rizvi NA, Hellmann MD, Snyder A, Kvistborg P, Makarov V, Havel JJ, et al. Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. Science 2015;348:124–8.
47. Chen PL, Roh W, Reuben A, Cooper ZA, Spencer CN, Prieto PA, et al. Analysis of immune signatures in longitudinal tumor samples yields insight into biomarkers of response and mechanisms of resistance to immune checkpoint blockade. Cancer Discov 2016;6:827–37.