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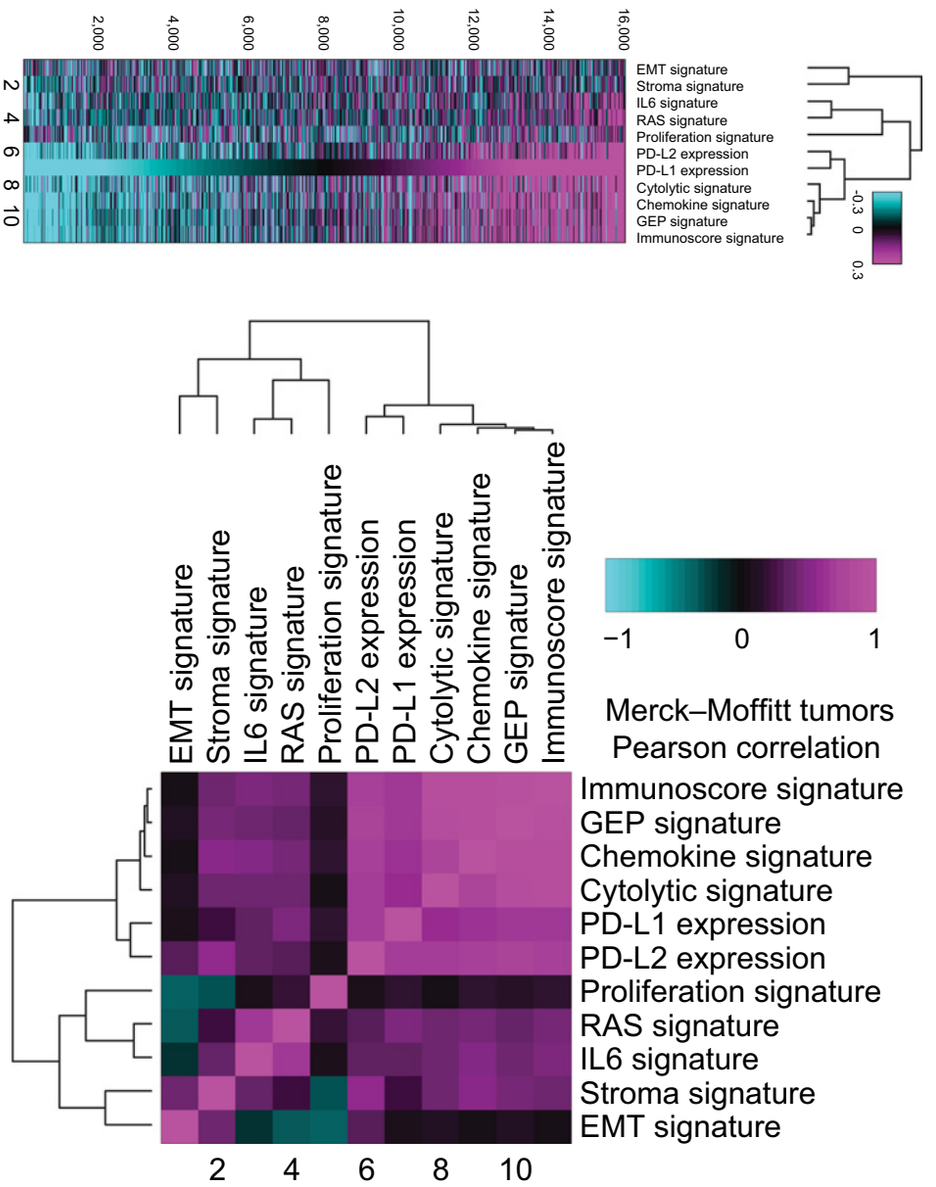

Figure 2.

Comparison of the PD-L1 mRNA expression obtained in this study with four published cytolytic signatures. A particularly strong correlation was found with those reported by Rooney and colleagues and Galon and colleagues. Magenta represents elevated gene expression (relative to the median) and positive correlation; cyan represents decreased gene expression (relative to the median) and negative correlation. EMT, epithelial–mesenchymal transition; GEP, T-cell–inflamed signature; IL, interleukin.

threshold for identifying the presence of tumors with potentially clinically relevant levels of PD-L1 gene expression. For each profiled sample or indication, the PD-L1 expression cut-off point for positivity was defined as the pan-cancer 75th percentile of PD-L1 expression in the Merck–Moffitt dataset. This pan-cancer cutoff was selected to be consistent with the point at which the distribution of PD-L1 expression in the Merck–Moffitt dataset deviates from a normal distribution (Supplementary Fig. S2), and the cutoff led the percentage of PD-L1–positive tumors to roughly align with response rates reported at the time of the analysis in melanoma, NSCLC, and renal cell carcinoma (RCC). It was also consistent with the cutoffs defined by the Youden index when comparing PD-L1 expression between colorectal microsatellite instability (MSI) and colorectal microsatellite stability (MSS), triple-negative breast cancers (TNBC), and luminal A breast cancers, and a combination of melanoma and NSCLC and remaining solid tumors in the Merck–Moffitt dataset (Supplementary Fig. S3).

Ranking of primary tumors using PD-L1 expression level

In Fig. 3A, the differences in PD-L1 expression are shown across the various tumor types for primary tumor samples included in the Merck–Moffitt dataset; this is reported as the percentage of tumors with PD-L1 positivity above the cutoff, as described in the Results. On the basis of this analysis, tumors could be grouped into 3 tiers: tier 1 (40%–60% of tumors), tier 2 (20%–40% of tumors), and tier 3 (<20% of tumors). The small overlap in confidence intervals across indications suggests that analysis of the Merck–Moffitt dataset is adequately powered to discriminate rankings in primary tumors based on the measurement of PD-L1 mRNA expression. In addition, the rankings of tumor types for metastatic tumor samples included

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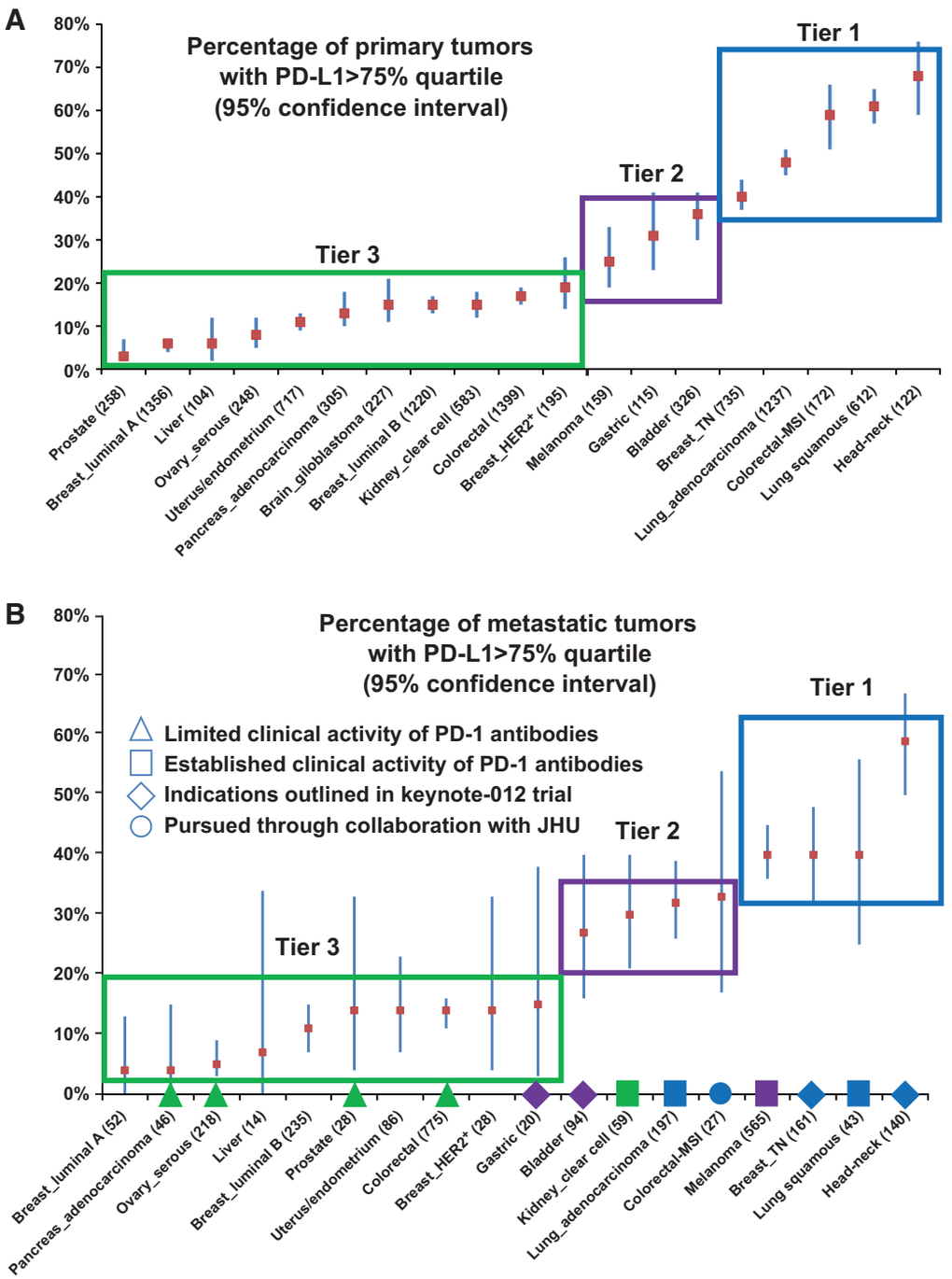

Figure 3.

Percentages of primary (**A**) and metastatic tumors (**B**) with high PD-L1 (>75th percentile; with 95% confidence intervals); Merck–Moffitt dataset. Only tumor types with >100 primary tumors available for analysis are shown. **A,** Three tiers of indications were identified for primary tumors; high levels of PD-L1 expression were found in 40% to 60% (tier 1), 20% to 40% (tier 2), and <20% (tier 3) of tumors, respectively. **B,** Symbols on the *x*-axis represent information at the time of analysis and the indications selected for the KEYNOTE-012 study; coloring of symbols indicate the tier to which the primary tumors belong. JHU, Johns Hopkins University; TN, triple negative.

in the Merck–Moffitt dataset were similar to those of primary tumors (Fig. 3B).

For 3 of the primary tumor sample types included in the Merck–Moffitt dataset, >50% of the samples were above the cutoff for PD-L1 positivity, as described in the Results; for 5 tumor types, ≥25% of samples exhibited high PD-L1 (Fig. 4). The highest proportion of samples above the 75th percentile for PD-L1 positivity was found for primary head and neck cancer (68%),

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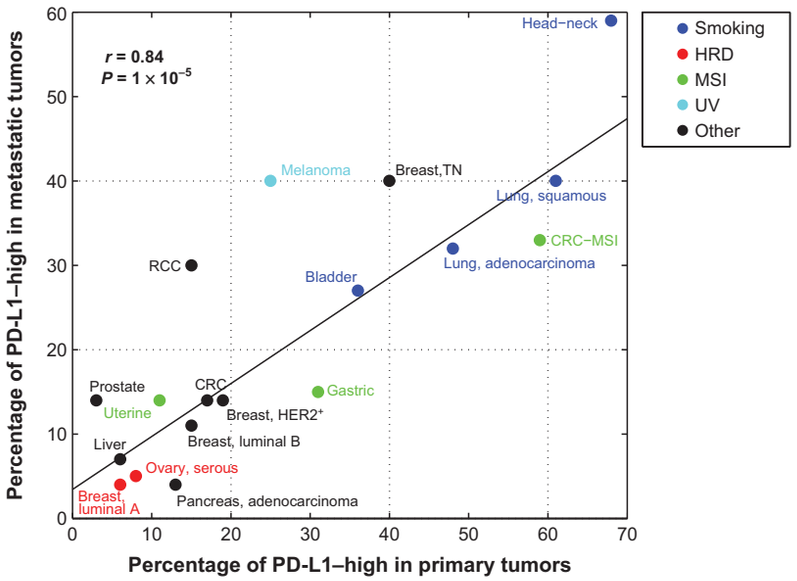

Figure 4.

Correlation of PD-L1–high prevalence in primary and metastatic tumors across different cancer types in Merck–Moffitt TCC ($P = 1 \times 10^{-5}$). CRC, colorectal cancer; HRD, homologous recombination deficiency; MSI-H, microsatellite instability–high; TCC, Total Cancer Care; TN, triple negative; UV, ultraviolet radiation.

followed by primary squamous cell lung cancer (61%), primary colorectal MSI (59%), and metastatic head and neck cancer (59%). The PD-L1 positivity threshold for primary adenocarcinoma lung cancer was 48%. Other tumors identified with high PD-L1 mRNA expression included primary and metastatic TNBC (40% for both), primary bladder cancer (36%), metastatic clear cell RCC (30%), and metastatic melanoma (40%). In contrast, HER2-positive, luminal A, and luminal B breast cancer subtypes had lower levels of PD-L1 mRNA expression than did TNBC (19%, 6%, and 15%, respectively, for primary and 14%, 4%, and 11%, respectively, for metastatic), as did primary/metastatic endometrial cancer (11%–14% above the positivity threshold).

The concordance of the percentages for primary compared with metastatic samples was significant ($R^2 = 0.84$; $P = 1.0 \times 10^{-5}$; Fig. 4). Although the concordance of PD-L1–positive prevalence between metastatic and primary tumors was very high, our analysis revealed some important differences; for example, RCC and melanoma have relatively higher percentages of PD-L1–positive expression in metastatic tumors than in primary tumors, whereas those for lung cancer are higher in primary tumors than in metastatic tumors (Fig. 4).

Additional molecular profiling of tumors not prevalent in the United States

Some large tumor datasets are biased toward tumor types that are prevalent in the United States and are underpowered for accurate molecular profiling of tumors that occur predominantly in other parts of the world. Gastric cancer is an important example of such a tumor type. Early access to a large cohort of gastric cancer samples from the Asian Cancer Research Group collaboration (19) allowed evaluation of the PD-L1 expression in that tumor type. The overall prevalence of PD-L1–positive gastric tumors based on the pan-cancer 75th percentile in the other tumor types was approximately 25%, which placed gastric cancer in the tier 2 group of indications for anti–PD-1 development (Fig. 3). Specific subsets were highly enriched for PD-L1 expression. As expected, MSI-high (MSI-H) tumors had particularly high levels of PD-L1 expression (Supplementary Fig. S3). In addition, some MSS PD-L1–high tumors are associated with Epstein–Barr virus (EBV) infection, and these were also associated with high levels of PD-L1 expression (Supplementary Fig. S4).

Association of indication ranking by PD-L1 activation with ranking by mutational load

A high level of neoantigens can lead to immune infiltration and subsequent PD-L1 activation (20); high mutation load produced by somatic mutations in a tumor might increase neoantigen expression and elevate PD-L1. This hypothesis was explored by determining the relationship between the percentages of tumors with elevated mutational load and elevated PD-L1 across tumor types, revealing a significant positive association (Fig. 5). Notably, MSI tumors (colon), which have the largest mutational load across all indications, also demonstrated the highest proportion of PD-L1 activation. The association between mutational load and PD-L1 or cytolytic activity is much weaker within indications (Supplementary Table S2) because the two measurements reflect related but largely orthogonal biological processes.

Impact of molecular profiling data on clinical trial design

The molecular profiling data shown in the results were taken into consideration in the development of KEYNOTE-012 (ClinicalTrials.gov, NCT01848834), a phase Ib, multicohort trial to evaluate pembrolizumab in patients with TNBC and head and neck, bladder, and gastric cancers that was initiated early in the pembrolizumab clinical development program. Patients were initially selected for PD-L1 positivity by use of IHC; subsequently, an expansion cohort in unselected patients with head and neck cancer was added. Clinical activity in PD-L1–unselected populations was confirmed in all 4 indications in this study; the ORRs and disease control rates were 19% and 44%, respectively, for cohort A TNBC (29), and the ORRs were 20%, 26%, and 22% for head and neck (30), bladder (31), and gastric cancers (32) for cohorts B, C, and D, respectively.

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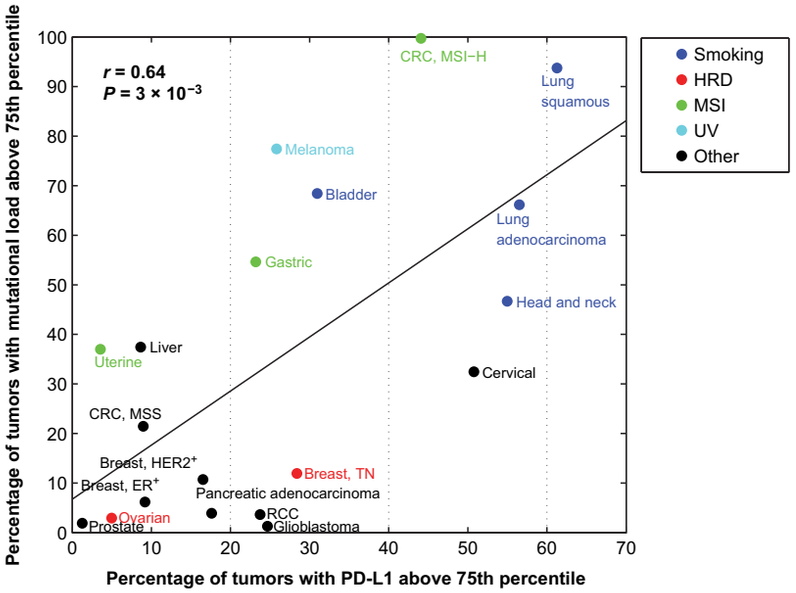

Figure 5.
Correlation between tumor mutation load and PD-L1 expression in TCGA dataset (https://tcga-data.nci.nih.gov/docs/publications/tcga/?). Each tumor type is colored according to its primary risk factor (smoking, HRD, MSI, UV, or "other"). CRC, colorectal cancer; ER^+, estrogen receptor positive; HRD, homologous recombination deficiency; MSS, microsatellite stable; TN, triple negative; UV, ultraviolet radiation.

Relative ranking between primary and metastatic tumors was generally consistent. Exceptions were clear cell RCC, which was ranked as tier 3 for primary tumors and tier 2 for metastatic tumors, and gastric cancer, which was ranked as tier 2 for primary tumors and tier 3 for metastatic tumors (Fig. 4). There is evidence of antitumor activity with PD-1/PD-L1 pathway inhibition in patients with metastatic RCC. In the open-label phase III CheckMate-025 trial, PD-1 inhibition with nivolumab as second- or third-line therapy improved the ORR over that achieved with the mTOR inhibitor everolimus (25% vs. 5%) in patients with advanced/metastatic clear cell RCC (33). The combination of nivolumab plus ipilimumab is also associated with improved responses over standard-of-care sunitinib in a large phase III trial of 1,096 patients with treatment-naïve advanced, clear cell RCC (34). Pembrolizumab is under investigation in this patient population as monotherapy (KEYNOTE-427, NCT02853344) and in combination with the tyrosine kinase inhibitor axitinib (KEYOTE-426, NCT02853331). In a phase Ib, nonrandomized dose-finding and dose-expansion trial, the combination of pembrolizumab and axitinib conferred an ORR of 73% in a cohort of 52 patients with previously untreated, advanced RCC who were unselected for PD-L1 expression (KEYNOTE-035, NCT02133742; ref. 35).

Response to pembrolizumab has been evaluated in the tier 3 indications in the basket KEYNOTE-028 trial, mostly in small patient populations with different disease states, including advanced, heavily pretreated, PD-L1–positive, estrogen receptor–positive, HER2-negative breast cancer ($n = 25$; ref. 36); pretreated, locally advanced/metastatic PD-L1–positive endometrial cancer ($n = 24$; ref. 37); advanced, PD-L1–positive ovarian cancer ($n = 26$; ref. 38); and advanced, treatment-resistant, PD-L1–positive colorectal cancer ($n = 23$; ref. 39). In line with the tier 3 ranking for primary tumors, antitumor activity for these indications seemed modest, with low ORRs of 12.0%, 13.0%, 11.5%, and 4.0%, respectively. The single patient who experienced objective (partial) response among the colorectal cancer cohort had MSI-H disease (39). In a study of patients with mismatch repair–deficient colorectal cancer and non-colorectal cancer tumors and patients with mismatch repair–proficient colorectal cancer, pembrolizumab antitumor activity was found for those with mismatch repair–deficient tumors but not mismatch repair–proficient colorectal cancer (40). Trials being actively pursued in other tier 3 indications include metastatic, castration-resistant prostate cancer (as monotherapy in KEYNOTE-199, NCT02787005; in combination therapies in KEYNOTE-365, NCT02861573), advanced hepatocellular carcinoma (KEYNOTE-224, NCT02702414), previously treated metastatic colorectal cancer (KEYNOTE-164, NCT02460198), and recurrent or metastatic gastric or gastroesophageal junction adenocarcinoma (KEYNOTE-059, NCT02335411).

Discussion

Large genomic datasets provide a unique resource for hypothesis-generating and hypothesis-validating molecular profiling. These sets are becoming essential bioinformatics tools for the development of precision medicine in oncology. In this study, analyses performed across large datasets of genomic profiles were used to steer indication for the anti–PD-1 inhibitor pembrolizumab. This molecular profiling of genomic cohorts allowed linkage of emerging clinical and PD-L1 IHC biomarker data from the pembrolizumab clinical development program with genomic profiles in large datasets based on biomarker prevalence data, biological contextualization using prior knowledge of tumor immune activation signatures, and ranking of tumors based on a gene expression score. This, in turn, enabled mapping of emerging clinical data to the biomarker data, setting a cutoff for positivity, and enabled development of proposals for new indications for clinical study. Although this dataset did not utilize PD-L1 IHC data, IHC scores were moderately correlated with PD-L1 mRNA expression (41).

Analysis of PD-L1 coexpression patterns revealed a highly significant association with a previously developed signature indicative of activation of cytolytic T cells (10). The cytolytic pattern was subsequently further investigated and elucidated

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by Galon and colleagues (6) and Rooney and colleagues (20), who determined that activation of T cells and natural killer cells is coupled with IFN and chemokine signaling. Moreover, a recently published analysis of mRNA profiles of baseline tumor samples from pembrolizumab-treated patients on a NanoString nCounter platform (NanoString Technologies, Inc.,) yielded an 18-gene, T-cell–inflamed immune signature that predicted response to pembrolizumab across several solid tumors. The predictive value of this signature, which contained IFN-responsive genes related to antigen presentation, chemokine expression, cytotoxic activity, and adaptive immune resistance, compared favorably with PD-L1 IHC in PD-L1–unselected populations (22). The finding that PD-L1 expression is positively associated with the cytolytic process is consistent with the role of PD-L1 as part of a negative feedback loop that is activated in the presence of cytolytic activity. Thus, activation of PD-L1 is a biomarker of robust cytolytic activity suppressed by a tumor-induced microenvironment. A strong correlation between the cause (cytolytic activity) and the negative feedback (activation of PD-L1 and PD-L2) suggests a state of equilibrium between tumor and immune system that, in some tumors, can be shifted by anti–PD-1 therapy toward antitumor activity.

Large datasets, such as those of Merck–Moffitt and TCGA, are well powered to accurately identify all the genes that play a role in cytolytic activation and concomitant negative feedback. The correlation of cytolytic genes with PD-L1 expression in these two large datasets and the almost perfect overlap between distinct large gene sets demonstrates a robust association between the cytolytic machinery and tumor PD-L1 overexpression. These datasets are also powered to accurately assess the distribution of PD-L1 activation scores for each tumor type and identify statistically significant differences in the scores among the tumors, particularly for metastatic tumors. The Merck–Moffitt dataset included profiling of a large number of metastatic tumors ($N \sim 4{,}000$) compared with the TCGA dataset, which notably lacks profiling of tumors in the metastatic setting in most indications. A similar ranking of indications was obtained in both datasets for primary tumors; however, notable exceptions in the ranking of primary versus metastatic tumors in the Merck–Moffitt dataset are RCC and melanoma, which have a significantly higher prevalence of PD-L1 positivity in metastatic lesions.

To further substantiate the biological rationale underlying indication selection, we demonstrated that ranking of tumors by mutational burden is consistent with ranking based on PD-L1 activation (Fig. 5). Mutational burden is one of the potential causal events underlying the cytolytic immune response and PD-L1 activation. Clinically validated indications at the time of our analyses, such as melanoma and NSCLC, and our candidate indications from tier 1 that were prioritized for clinical development, such as head and neck, bladder, and MSI-H colon cancers, not only have high levels of PD-L1 activation, but also have high rates of mutational burden. In gastric tumors, the observed PD-L1 activation was caused by not only mutational burden (MSI), but also EBV infection. The presence of viral infections, such as human papillomavirus and EBV, may explain PD-L1 activation in cervical tumors and in subsets of head and neck tumors.

The strong relationship between mutational rate and elevated PD-L1 expression is true from the point of view of a pan-cancer analysis but not when the relationship is studied in individual indications (except for MSI cancers; Supplementary Table S2). This is potentially because of the heterogeneity of causal events for cytolytic immune response and tumor escape mechanisms, which decouple the presence of neoantigens from the presence of cytolytic infiltrate and PD-L1 activation over the long-term evolution of the tumor.

Molecular profiling also guided the clinical development of pembrolizumab in tier 3 lower ranked tumor types. It was predicted that the likelihood of observing any meaningful clinical monotherapy activity in the PD-L1–unselected population from those indications would be low; therefore, a biomarker-based enrichment strategy was used. This led to the initiation of an ongoing, multicohort, nonrandomized phase Ib basket clinical trial (KEYNOTE-028, NCT02054806) of pembrolizumab monotherapy (10 mg/kg dosed every 2 weeks) in more than 450 patients across 20 different tumor types. The basket trial design allowed for the clinical evaluation of tumors from different tumor types in a single study. In KEYNOTE-028, patients were enrolled whose tumors expressed elevated levels of PD-L1, but not as high as the cutoff selected to separate tier 1 tumors from tier 3 tumors. The exact levels of PD-L1 expression necessary to achieve substantial response rates among tier 3 tumor types remain to be determined.

Pembrolizumab may be active as monotherapy for the biomarker-selected patients in tier 3 indications, and its use in conjunction with other therapies in these indications may be even more effective. Indeed, pembrolizumab in combination with pemetrexed and carboplatin is already approved by the FDA as a first-line option for metastatic nonsquamous NSCLC (13), and multiple clinical trials are under way in which combination therapy with pembrolizumab and a broad range of agents are being tested for initial safety and efficacy across indications, including those in tier 3. In this rapidly evolving field, many hypotheses regarding combination activity will be clinically tested, ranging from specific immune-based combination mechanisms to broadly orthogonal approaches with standard chemotherapies and newer targeted therapies. The results of such trials must be evaluated to understand the molecular basis for successful and unsuccessful combination therapies. In combination with the results of ongoing clinical trials, a deeper understanding of tumor molecular subtypes and characteristics of immune infiltration will potentially offer insight into mechanisms of resistance to pembrolizumab and approaches to overcoming that resistance.

As a testimony of the validity of our analytic approach and the value of molecular databases to drive clinical development, pembrolizumab has been approved in 3 of the 4 indications prioritized for rapid clinical development as a result of the effort described in this article (head and neck, urothelial carcinoma, and gastric cancer; Supplementary Table 1).

In conclusion, the strategy of using molecular profiling of PD-L1 expression in a large dataset of gene expression profiles of human tumors has enabled rapid expansion of pembrolizumab development into new indications and has been validated by the significant levels of clinical activity subsequently reported for those indications.

Disclosure of Potential Conflicts of Interest

M. Ayers holds ownership interest (including patents) in Merck. T.K. McClanahan holds ownership interest (including patents) in Merck.

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R.F. Perini holds ownership interest (including patents) in Merck. J. Cheng holds ownership interest (including patents) in Merck. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Nebozhyn, J. Cheng
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Ayers, M. Nebozhyn, R. Cristescu, R.F. Perini, J. Cheng, D.R. Kaufman, A. Loboda
Writing, review, and/or revision of the manuscript: M. Ayers, M. Nebozhyn, R. Cristescu, T.K. McClanahan, R.F. Perini, E.H. Rubin, J. Cheng, D.R. Kaufman, A. Loboda
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Nebozhyn, R. Cristescu
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