Genomic correlates of response to CTLA4 blockade in metastatic melanoma

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Monoclonal antibodies directed against cytotoxic T lymphocyte–associated antigen-4 (CTLA-4), such as ipilimumab, yield significant clinical benefit for patients with metastatic melanoma by inhibiting immune checkpoint activity, but clinical predictors of response to these therapies remain incompletely characterized. To investigate the roles of tumor-specific neoantigens and alterations in the tumor microenvironment in the response to ipilimumab, we analyzed whole exomes from pretreatment melanoma tumor biopsies and matching germline tissue samples from 110 patients. For 40 of these patients, we also obtained and analyzed transcriptome data from the pretreatment tumor samples. Overall mutational load, neoantigen load, and expression of cytolytic markers in the immune microenvironment were significantly associated with clinical benefit. However, no recurrent neoantigen peptide sequences predicted responder patient populations. Thus, detailed integrated molecular characterization of large patient cohorts may be needed to identify robust determinants of response and resistance to immune checkpoint inhibitors.

Blockade of the T cell inhibitory receptor cytotoxic T lymphocyte antigen-4 (CTLA-4) with the monoclonal antibody ipilimumab yields improvements in overall survival in patients with metastatic melanoma as a monotherapy (1, 2) or in combination with other T cell immune checkpoint inhibitors (3, 4). Although overall single-agent response rates are low, a long-term clinical benefit is consistently observed for approximately 20% of treated patients (5, 6). Preclinical and clinical studies have suggested that tumor-specific missense mutations may generate individual neoantigens that mediate response to ipilimumab and other immune checkpoint inhibitors (7–10). Clinical studies of exceptional responders (II) and of small cohorts of melanoma patients have highlighted NRAS mutation status, total neoantigen load, and a neoantigen-derived tetrapeptide signature as possible correlates of response to ipilimumab in metastatic melanoma (12, 13). RNA-based studies have also identified gene expression signatures linked to immune infiltration within the tumor microenvironment that correlate with overall survival, neoantigen load (14, 15), and resistance to immunotherapy (16). To date, however, comprehensive genomic studies of tumor- and immune-related factors in larger (i.e., >100 patients) clinical cohorts have not been reported.

We hypothesized that both tumor-specific neoantigens and the tumor immune microenvironment might influence clinical benefit from ipilimumab. To test this, we performed whole exome sequencing on a cohort of 110 patients with metastatic melanoma from whom pretreatment tumor biopsies were available for study (Fig. 1A). Tu-
sequencing. This cohort included 92 cutaneous, 4 mucosal, and 14 occult melanomas. After whole exome sequencing (WES) of matched tumor and germline samples (17), quality control metrics were applied to ensure sensitive mutation detection (18). Average exome-wide target coverage was 183.7-fold for tumor samples and 157.2-fold for germline samples. Somatic mutation identification (table S1) and germline HLA typing (table S2) were performed using established methods (14, 19). The median nonsynonymous mutational load was 197 per sample (range: 7 to 5854), which is consistent with the known high mutational loads in cutaneous melanoma (I3, 20).

To stratify our cohort, “clinical benefit” was defined using a composite endpoint of complete response or partial response to ipilimumab by RECIST criteria (21); or stable disease by RECIST criteria with overall survival greater than 1 year (n = 27). “No clinical benefit” was defined as progressive disease by RECIST criteria or stable disease with overall survival less than 1 year (n = 73). The basis for these designations stems from clinical trials demonstrating that ipilimumab significantly improves median overall survival (OS), with a subset of patients surviving beyond 2 years (~20%), but does not impact progression-free survival (PFS) (5, 22). A separate group of 10 patients showed early progression on ipilimumab (PFS < 6 months) but their overall survival patterns exceeded 2 years; these patients were considered as a separate patient subgroup (Fig. 1B and tables S2 and S3).

Overall, nonsynonymous mutational load was significantly associated with clinical benefit from ipilimumab (P = 0.0076; Mann-Whitney) (Fig. 2A). This result confirms previous findings for ipilimumab in melanoma (19) and is consistent with observations regarding response to other immune checkpoint inhibitors in cancer (23, 24). Clinical metrics such as patient age, gender, tumor histology, primary tumor site, number of therapies received prior to ipilimumab, and lactate dehydrogenase (LDH) levels at initiation of ipilimumab monotherapy showed no significant correlation with clinical response to ipilimumab in this cohort (p > 0.05 for all) (table S3). The long-term survival subset tracked with the no clinical benefit subset in terms of mutational load, but the sample size in this group was too small to draw definitive conclusions (Fig. 2A). No genes were enriched for nonsynonymous mutations in the clinical benefit or no clinical benefit subgroups, including BRAF and NRAS (figs. S1 and S2).

Next, we sought to determine the relationship between neoantigen load and clinical benefit to ipilimumab. First, we identified putative immunogenic 9- and 10-amino acid neoantigens with ≤ 500 nM binding affinity for HLA class I molecules across the cohort using patient-specific nonsynonymous mutations and HLA types (tables S2 and S4) (14). The median predicted neoantigen load was 369 neoantigens per sample (range: 9-14,880). Overall, neoantigen load was significantly associated with clinical benefit (p = 0.027; Mann-Whitney) (Fig. 2A), although high neoantigen load outliers among nonresponders and low neoantigen load outliers among responders were observed. Neoantigen load was strongly correlated with mutational load (Spearman’s rho = 0.97, P < 0.0001), as expected given that neoantigens often arise from nonsynonymous mutations. The association between neoantigen load and clinical benefit remained significant when randomly re-distributed HLA types were used to infer putative neoantigen binders (P = 0.0096; Mann-Whitney) (fig. S3)

We then sought to determine the association between aggregate neoantigen properties and clinical benefit. The correlation between neoantigen load and clinical benefit diminished when applying increasingly stringent thresholds for affinity of binding (P = 0.034, 0.039, and 0.042 for affinity thresholds of < 250 nM, 100 nM, and 50 nM, respectively; Mann-Whitney) (Fig. 2B). We also applied multivariate models that controlled for prior RAF inhibitor treatment and M class at start of therapy (18). These analyses confirmed that patients with high neoantigen or mutational loads (>100) were significantly more likely to have clinical benefit from ipilimumab (P = 0.0371 and P = 0.0169, respectively).

We then investigated whether any recurrent neoantigens or neoantigen epitopes might predict ipilimumab response. Of the 75,179 unique neoantigens in our cohort, 28 (~0.04%) were found in >1 clinical benefit patient but were absent in all no clinical benefit or long term survival patients (Table 1). Examination of these recurrent neoantigens did not reveal any shared features or features exclusive to responders. Previously described immunogenic nonamers identified in patients who achieved clinical benefit from immune checkpoint blockade were not observed in any patient within this cohort, including several that have undergone experimental validation (7, 11, I3, 23). Furthermore, a tetrapeptide signature (13) previously associated with ipilimumab response was not enriched in the clinical benefit cohort relative to the no clinical benefit cohort (fig. S4) (25). Thus, the vast majority of clinical benefit-associated neoantigens and neoantigen epitopes identified through DNA sequencing appear to be private events without obvious recurrent features.

To evaluate neoantigens most likely to engender an immune response, we next examined whether predicted immunogenic neoantigens were expressed in mutated tumors. Here, we leveraged paired DNA and RNA sequencing data obtained for 40 patients (13 clinical benefit, 22 no clinical benefit, 5 long term survival) from whom high-quality archival formalin-fixed, paraffin-embedded (FFPE) tumor tissue was available (18). Among these patients, the median number of predicted neoantigens using nonsynonymous mutations and HLA-typing alone was 395/patient (range: 12
to 6984). Filtering these putative neoantigens using patient-matched transcriptome data decreased the median neoantigen load to 198/patient (range: 4 to 4622). A similar filtering approach that utilized unmatched melanoma gene expression data from the Cancer Genome Atlas (18, 26) also eliminated neoantigens unlikely to be expressed (in this case, the median neoantigen load was 347/patient, range: 12 to 6159); however, the remaining neoantigens only partially overlapped those that were inferred using patient-matched RNA (Fig. 2, C and D).

Conceivably, an inadequate immune response to tumor neoantigens could also be explained by aberrant HLA class I expression, which has been previously observed in melanoma (27). However, we found that HLA class I genes were expressed in all patients and at similar levels across response groups (fig. S5, A and B). Moreover, somatic mutations in HLA class I genes were rare in this cohort and did not segregate by response group (table S5). Therefore, matched genome and transcriptome sequencing appeared to improve the identification of patient-specific neoantigens, which were not impacted by aberrant HLA expression or mutation status.

Finally, we leveraged the total transcriptome data from this cohort (42 patients total) to characterize an RNA expression profile in the tumor microenvironment previously associated with immune infiltrate. Specifically, the geometric mean of expression of granzyme A (GZMA) and perforin (PRF1) has been shown to correlate with both the cytolytic activity of local immune infiltrates and the neoantigen load (14, 15). Both genes were significantly enriched in the ipilimumab clinical benefit cohort compared to the no clinical benefit cohort (P = 0.042; Mann-Whitney) (Fig. 3A). Also, the long term survival cohort expressed these cytolytic genes at levels similar to the clinical benefit group. Interestingly, the expression of CTLA-4 itself and the peripheral T cell inhibitory protein programmed cell death 1 ligand 2 (PD-L2) were also significantly elevated in the clinical benefit cohort compared to the no clinical benefit cohort (P = 0.033 and P = 0.041; Mann-Whitney) (Fig. 3B). Levels of expression of HLA class I were similar across clinical response groups (P > 0.05 for all; Mann-Whitney) (Fig. 3C). Together, these findings suggest that expression of immune checkpoint molecules themselves may correlate with immunotherapy response.

Overall, our findings imply that both DNA- and RNA-level genomic information may have predictive value for ipilimumab-based therapy. In contrast, recurrent neoantigens (e.g., those occurring in more than one ipilimumab responder) were rare in our cohort—and those that were observed were not necessarily matched by HLA type. These results suggest that the discovery of specific or recurrent neoantigens that mediate response to immunotherapy may require patient cohorts that are much larger in size than those currently available, especially given the importance of HLA restriction in mediating neoantigen recognition (10). Incorporation of patient-matched RNA-level information may help prioritize putative neoantigens and elucidate possible tumor immune microenvironmental effects, including IFN-related genes and additional immune checkpoint molecules that are relevant in melanoma (28). Additionally, prediction of neoantigens originating from fusion products and class II neoantigens may further inform genomic correlates of response (29–31).

In this study, we observed a distinct set of patients who experienced long survival following treatment despite early progression of disease. Patterns of initial increases in tumor mass or delayed responses to therapy have been observed following cancer immunotherapy in the past (32). Thus, these observations raise the possibility that neoantigen loads or tumor cytolytic and immune checkpoint expression may play meaningful functional roles in this context, although these findings require further exploration in larger clinical cohorts. Additional studies of clinical response and resistance to immune checkpoint inhibitors may benefit from integrating exome and transcriptome sequencing data to inform the relative contributions of tumor immunogenicity and host immune infiltration in determining clinical benefit.

REFERENCES AND NOTES


Materials and methods are available as supplementary materials on Science online.
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SUPPLEMENTARY MATERIALS
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Materials and Methods
Figs. S1 to S5
Tables S1 to S5

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**Fig. 1. Study design and clinical stratification.**

(A) 150 patients were identified for whole-exome sequencing of tumor and germline DNA. To be included in the original clinical cohort, patients had to have received ipilimumab monotherapy for metastatic cutaneous melanoma, have pretreatment germline and tumor samples available for sequencing, and have had overall survival for >14 days following initiation of ipilimumab therapy. 110 patients were eventually included in analysis after exclusions due to inadequate post-sequencing quality control (n = 40) (18). Manual review of raw sequencing data was performed to exclude samples with evidence suggesting low purity, high contamination by ContEst (33), or discordant copy number quality control. 62 patients, including 2 who failed DNA quality-control, had FFPE tumor samples available for transcriptome sequencing. After manual review for post-RNA-sequencing quality control, 42 samples were additionally analyzed for tumor microenvironment signatures, and 40 with matched whole exome sequencing were analyzed for neoantigen expression (14). (B) Patients were stratified into response groups based on RECIST criteria (21) (CR = complete response, PR = partial response, SD = stable disease, PD = progressive disease, MR = mixed response), duration of overall survival (OS), and duration of progression-free survival (PFS). All two-way comparisons were done comparing patients who achieved clinical benefit with ipilimumab (CR or PR by RECIST criteria or OS > 1 year with SD by RECIST criteria) (n = 27) to those with minimal or no benefit from ipilimumab (PD by RECIST criteria or OS < 1 year with SD by RECIST criteria) (n = 73). An additional cohort of patients who achieved long-term survival (OS > 2 years) following ipilimumab treatment with early tumor progression (PFS < 6 months) were considered separately (n = 10).
Fig. 2. Overall mutational load, overall neoantigen load, and expression-based neoantigen analysis as predictors of response to ipilimumab. (A) Elevated nonsynonymous mutational load and neoantigen load are associated with response to ipilimumab ($P = 0.0076$ and $0.027$, respectively). An additional 20 points are not shown due to outlying high neoantigen loads in a subset of patients. (B) No trend in increased significance is observed when comparing burden of higher-affinity neoantigens with respect to response to ipilimumab. Lower IC50 values on the x-axis imply stronger HLA binding affinity ($P = 0.027$ for affinity < 500 nM; $P = 0.034$ for affinity < 250 nM; $P = 0.038$ for affinity < 100 nM; $P = 0.042$ for affinity < 50 nM). An additional 34 points are not shown due to outlying high neoantigen loads in a subset of patients. (C) A sample size of 40 patients with complete DNA-sequencing, RNA-sequencing, and clinical annotation was insufficient to discern significant differences in neoantigen load or expressed neoantigen load among response cohorts, but a trend was observed for increased neoantigen load among clinical benefit patients compared to no clinical benefit patients ($P > 0.05$ for all). (D) Patient-specific RNA-seq provides distinct information on tumor gene expression compared to TCGA melanoma data from a separate patient cohort. While TCGA and RNA-seq data agree on the expression of the majority of neoantigens ($n = 12,316$) for 40 patients who had high-quality DNA- and RNA-sequencing data available for neoantigen and gene expression analysis, TCGA expression data overestimates the number of neoantigens expressed by 6320 in this patient cohort, and 166 neoantigens that are expressed by patient tumors would be missed by TCGA filtering alone. Additionally, a large proportion of neoantigens ($n = 4349$) are expressed at negligible levels in patient tumors. Asterisks indicate $P < 0.05$. 
Fig 3. Immune microenvironment cytolytic and immune activity correlates with response to ipilimumab. (A) Patients who achieved clinical benefit from immune checkpoint blockade therapy had significantly higher levels of tumor cytolytic activity than those who had minimal or no benefit from ipilimumab ($P = 0.039$). (B) Patients who achieved clinical benefit from ipilimumab therapy had significantly higher levels of expression immune checkpoint receptors than those who did not (CTLA4: $P = 0.033$, PD-L2: $P = 0.041$). One point is not shown due to an outlying high CTLA4 expression value in a nonresponder patient (>50 RPKM). (C) Response to ipilimumab did not correlate with expression of or mutations in HLA alleles ($P > 0.05$ for all). Asterisks indicate $P < 0.05$. 
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Table 1. Recurrent neoantigens identified exclusively in the clinical benefit cohort with associated HLA type. Variants were manually reviewed in Integrated Genomics Viewer (24).