ORIGINAL ARTICLE

Genetic Basis for Clinical Response to CTLA-4 Blockade in Melanoma

Alexandra Snyder, M.D., Vladimir Makarov, M.D., Taha Merghoub, Ph.D.,
Jianda Yuan, M.D., Ph.D., Jesse M. Zaretsky, B.S., Alexis Desrichard, Ph.D.,
Logan A. Walsh, Ph.D., Michael A. Postow, M.D., Phillip Wong, Ph.D.,
Teresa S. Ho, B.S., Travis J. Hollmann, M.D., Ph.D., Cameron Bruggeman, M.A.,
Kasthuri Kannan, Ph.D., Yanyun Li, M.D., Ph.D., Ceyhan Elipenahli, B.S.,
Cailian Liu, M.D., Christopher T. Harbison, Ph.D., Lisu Wang, M.D.,
Antoni Ribas, M.D., Ph.D., Jedd D. Wolchok, M.D., Ph.D.,
and Timothy A. Chan, M.D., Ph.D.

ABSTRACT

BACKGROUND

Immune checkpoint inhibitors are effective cancer treatments, but molecular determinants of clinical benefit are unknown. Ipilimumab and tremelimumab are antibodies against cytotoxic T-lymphocyte antigen 4 (CTLA-4). Anti–CTLA-4 treatment prolongs overall survival in patients with melanoma. CTLA-4 blockade activates T cells and enables them to destroy tumor cells.

METHODS

We obtained tumor tissue from patients with melanoma who were treated with ipilimumab or tremelimumab. Whole-exome sequencing was performed on tumors and matched blood samples. Somatic mutations and candidate neoantigens generated from these mutations were characterized. Neoantigen peptides were tested for the ability to activate lymphocytes from ipilimumab-treated patients.

RESULTS

Malignant melanoma exomes from 64 patients treated with CTLA-4 blockade were characterized with the use of massively parallel sequencing. A discovery set consisted of 11 patients who derived a long-term clinical benefit and 14 patients who derived a minimal benefit or no benefit. Mutational load was associated with the degree of clinical benefit (P=0.01) but alone was not sufficient to predict benefit. Using genomewide somatic neoepitope analysis and patient-specific HLA typing, we identified candidate tumor neoantigens for each patient. We elucidated a neoantigen landscape that is specifically present in tumors with a strong response to CTLA-4 blockade. We validated this signature in a second set of 39 patients with melanoma who were treated with anti–CTLA-4 antibodies. Predicted neoantigens activated T cells from the patients treated with ipilimumab.

CONCLUSIONS

These findings define a genetic basis for benefit from CTLA-4 blockade in melanoma and provide a rationale for examining exomes of patients for whom anti–CTLA-4 agents are being considered. (Funded by the Frederick Adler Fund and others.)

From the Department of Medicine (A.S., T.M., M.A.P., J.D.W.), Human Oncology and Pathogenesis Program (A.S., V.M., A.D., L.A.W., K.K., T.A.C.), Swim across America–Ludwig Collaborative Research Laboratory (T.M., Y.L., C.E., C.L., J.D.W.), Department of Radiation Oncology (T.A.C.), Department of Pathology (T.J.H.), and Immunology Program, Ludwig Center for Cancer Immunotherapy (J.Y., P.W., T.S.H., J.D.W.), Memorial Sloan Kettering Cancer Center; Weill Cornell Medical College (A.S., M.A.P., J.D.W., T.A.C.); and Department of Mathematics, Columbia University (C.B.) — all in New York; the Department of Molecular and Medical Pharmacology (J.M.Z., A.R.) and the Department of Medicine, Division of Hematology–Oncology, Jonsson Comprehensive Cancer Center (A.R.), University of California, Los Angeles, Los Angeles; and Bristol-Myers Squibb, Princeton, NJ (C.T.H., L.W.). Address reprint requests to Dr. Chan at the Human Oncology and Pathogenesis Program, Memorial Sloan Kettering Cancer Center, 1275 York Ave., Box 20, New York, NY 10065, or at chant@mskcc.org; or to Dr. Wolchok at the Ludwig Center for Cancer Immunotherapy, Memorial Sloan Kettering Cancer Center, 1275 York Ave., New York, NY 10065, or at wolchokj@mskcc.org.

Drs. Snyder, Makarov, Merghoub, and Yuan and Drs. Wolchok and Chan contributed equally to this article.

IMMUNE CHECKPOINT BLOCKADE HAS LED to durable antitumor effects in patients with metastatic melanoma, non–small-cell lung cancer, and other tumor types, but the factors determining whether a patient will have a response remain elusive.[1,2] The fully human monoclonal antibodies ipilimumab and tremelimumab block cytotoxic T-lymphocyte antigen 4 (CTLA-4), resulting in T-cell activation. Some studies have established correlations between outcomes with ipilimumab and peripheral-blood lymphocyte count, markers of T-cell activation,[3] an "inflammatory" microenvironment,[4,5] and maintenance of high-frequency T-cell receptor clonotypes.[6]

The relationship among the genomic landscape of the tumor, the mutational load, and the benefit from treatment remains obscure. The immunogenicity resulting from nonsynonymous melanoma mutations has been shown in a mouse model,[7] and the antigenic diversity of human melanoma tumors has been modeled in silico[8] and in melanoma-specific CD8 T-cell responses after treatment with ipilimumab.[9] Effector and helper T-cell function and regulatory T-cell depletion are necessary for the efficacy of CTLA-4 blockade,[10] but there is not an association between a specific HLA type and a clinical benefit.[11] Melanomas have very high mutational burdens (0.5 to >100 mutations per megabase) as compared with other solid tumors.[12] Elegant studies have shown that somatic mutations can give rise to neoepitopes[13] and that these may serve as neoantigens.[14-16] We conducted a study to determine whether the genetic landscape of a tumor affects the clinical benefit provided by CTLA-4 blocking agents.

METHODS

SAMPLE ACQUISITION AND DNA PREPARATION

For the discovery set, we conducted whole-exome sequencing of DNA from tumors and matched normal blood from 25 ipilimumab-treated patients. A validation set included an additional 39 patients, of whom 5 were treated with tremelimumab. Primary tumor samples and matched normal peripheral-blood specimens were obtained after the patients had provided written informed consent. DNA was extracted, and exon capture was performed with the use of the SureSelect Human All Exon 50-Mb kit (Agilent Technologies). Enriched exome libraries were sequenced on the HiSeq 2000 platform (Illumina) to provide a mean exome coverage of more than 100× (Memorial Sloan Kettering Cancer Center Genomics Core and Broad Institute).

IMMUNOGENICITY ANALYSIS OF SOMATIC MUTATIONS

We created a bioinformatic tool to translate all mutations in exomes and then evaluate binding with major histocompatibility complex (MHC) class I molecules. The neoantigen signature was generated from the nonamers containing four amino acid strings of peptides that are common to tumors from patients with a long-term benefit from therapy. Details are provided in the Supplementary Appendix, available with the full text of this article at NEJM.org.

INTRACELLULAR CYTOKINE STAINING

Candidate neoantigen peptides were synthesized (GenScript), cultured with autologous peripheral-blood mononuclear cells (PBMCs), and then analyzed by means of intracellular cytokine staining for interleukin-2, CD107a, macrophage inflammatory protein 1β, tumor necrosis factor α, and interferon-γ on restimulation of cells with the candidate peptides.

STATISTICAL ANALYSIS

The Mann–Whitney test was used to compare mutational loads, and the log-rank test was used to compare Kaplan–Meier curves. The statistical methods used in the study are more fully described in the Supplementary Appendix.

RESULTS

MUTATIONAL LANDSCAPE OF MELANOMAS FROM THE STUDY PATIENTS

Baseline patient characteristics are shown in Table 1 (for more detailed information, see Tables S1 and S2 in the Supplementary Appendix). The study involved patients with and those without a long-term clinical benefit from therapy (CTLA-4 blockade alone or CTLA-4 blockade with resection of an isolated stable or nonresponding lesion). A long-term clinical benefit was defined by radiographic evidence of freedom from disease or evidence of a stable or decreased volume of disease for more than 6 months. Lack of a long-term ben-
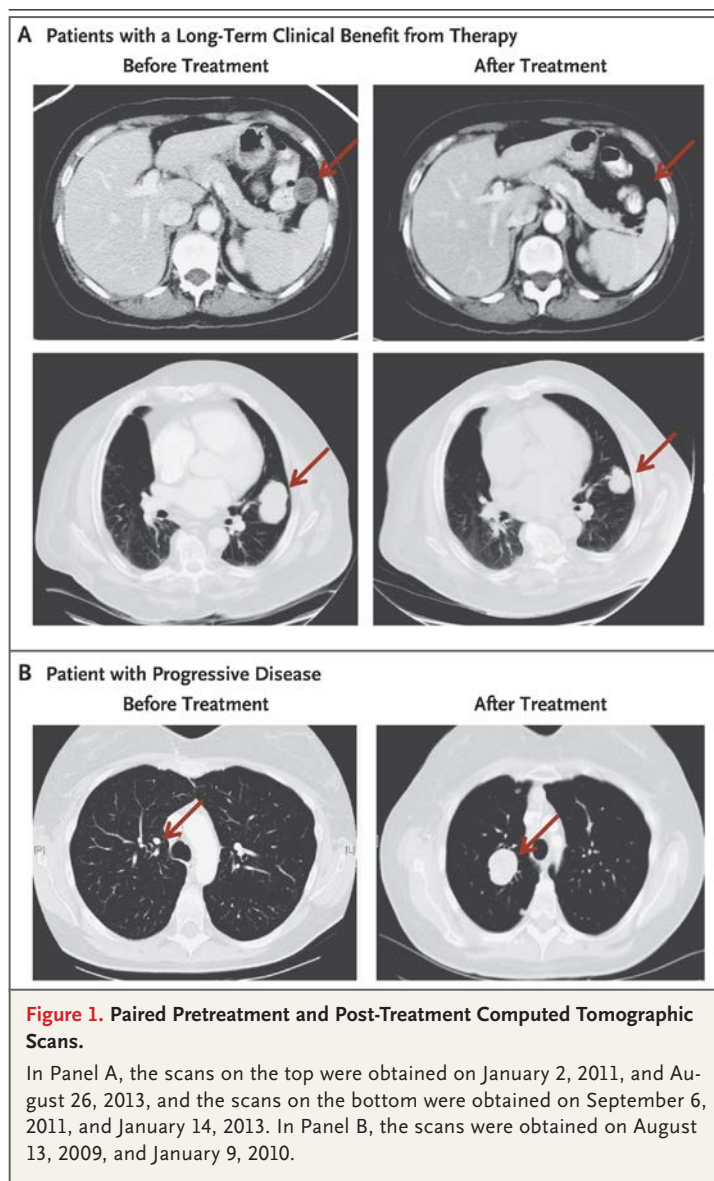
Table 1. Clinical Characteristics of the Patients in the Discovery and Validation Sets, According to Clinical Benefit from Therapy.				
Characteristic	Discovery Set		Validation Set	
	Long-Term Benefit (N=11)	Minimal or No Benefit (N=14)	Long-Term Benefit (N=25)	Minimal or No Benefit (N=14)
Age at start of treatment — yr				
Median	63	60	66	57
Range	39–70	48–79	33–90	18–74
Sex — no. of patients (%)				
Female	3 (27)	8 (57)	9 (36)	5 (36)
Male	8 (73)	6 (43)	16 (64)	9 (64)
Disease origin — no. of patients (%)				
Acral	0	3 (21)	1 (4)	1 (7)
Uveal	0	0	1 (4)	0
Cutaneous	10 (91)	8 (57)	15 (60)	11 (79)
Unknown primary	1 (9)	3 (21)	3 (12)	0
Not available	0	0	5 (20)	2 (14)
BRAF or NRAS mutation — no. of patients (%)				
No	1 (9)	6 (43)	17 (68)	11 (79)
Yes	10 (91)	8 (57)	8 (32)	3 (21)
Lactate dehydrogenase level at start of therapy — no. of patients (%)				
Normal	8 (73)	8 (57)	8 (32)	9 (64)
Above normal	2 (18)	5 (36)	3 (12)	3 (21)
Not available	1 (9)	1 (7)	14 (56)	2 (14)
Duration of response to therapy — wk				
Median	59	14	130	11
Range	42–361	11–23	64–376	3–29
Previous therapies — no.*				
Median	1	1	0	0
Range	0–3	0–2	0–2	0–3
Melanoma stage at time of diagnosis — no. of patients (%)				
IIIC	0	0	3 (12)	0
M1a	0	1 (7)	4 (16)	1 (7)
M1b	5 (45)	1 (7)	2 (8)	3 (21)
M1c	6 (55)	12 (86)	16 (64)	10 (71)
Overall survival — yr†				
Median	4.4	0.9	3.3	0.8
Range	2.0–6.9	0.4–2.7	1.6–7.2	0.2–2.1

* Previous therapies included interleukin-2 and cytotoxic chemotherapy.
† Overall survival was calculated from the date of the first dose of ipilimumab to the date of death or censoring of data.

Figure 1. Paired Pretreatment and Post-Treatment Computed Tomographic Scans.

In Panel A, the scans on the top were obtained on January 2, 2011, and August 26, 2013, and the scans on the bottom were obtained on September 6, 2011, and January 14, 2013. In Panel B, the scans were obtained on August 13, 2009, and January 9, 2010.

efit was defined by tumor growth on every computed tomographic scan after the initiation of treatment (no benefit) or a clinical benefit lasting 6 months or less (minimal benefit). Representative scans are shown in Figure 1, and Figure S1 in the Supplementary Appendix.

To determine the genetic features associated with a sustained benefit from CTLA-4 blockade, we analyzed DNA in tumor and matched blood samples using whole-exome sequencing. In the discovery set, we generated 6.4 Gb of mapped sequence, with more than 99% of the target sequence covered to at least 10× depth and a mean exome coverage of 103× (Table S3 and Fig. S2 in

the Supplementary Appendix). The wide ranges of mutational burdens (Fig. 2A, and Table S3 in the Supplementary Appendix) and recurrent and driver mutations (Fig. S2C and S2D and Table S4 in the Supplementary Appendix) among samples were consistent with previously reported findings.[17-19] The ratio of transitions to transversions (Fig. S2E in the Supplementary Appendix) and the frequency of nucleotide changes (Fig. S2F in the Supplementary Appendix) were similar in the discovery and validation sets.[12] No gene was universally mutated across patients with a sustained benefit.

ASSOCIATION BETWEEN MUTATIONAL BURDEN AND CLINICAL BENEFIT

We hypothesized that an increased mutational burden in metastatic melanoma samples would correlate with a benefit from CTLA-4 blockade. There was a significant difference in mutational load between patients with a long-term clinical benefit and those with a minimal benefit or no benefit, both in the discovery set (P=0.01 by the Mann–Whitney test) and in the validation set (P=0.009 by the Mann–Whitney test) (Fig. 2A, and Table S5 in the Supplementary Appendix). In the discovery set, a high mutational load was significantly correlated with improved overall survival (P=0.04 by the log-rank test) (Fig. 2B), and there was a trend toward improved survival in the validation set (Fig. S3A in the Supplementary Appendix). The latter set included eight patients with nonresponding tumors who otherwise had systemic disease control, which may confound the relationship between mutational load and survival. Further subdivision into four clinical categories was suggestive of a dose–response relationship in the discovery set (Fig. S3B in the Supplementary Appendix). These data indicate that a high mutational load correlates with a sustained clinical benefit from CTLA-4 blockade but that a high load alone is not sufficient to impart a clinical benefit, because there were tumors with a high mutational burden that did not respond to therapy.

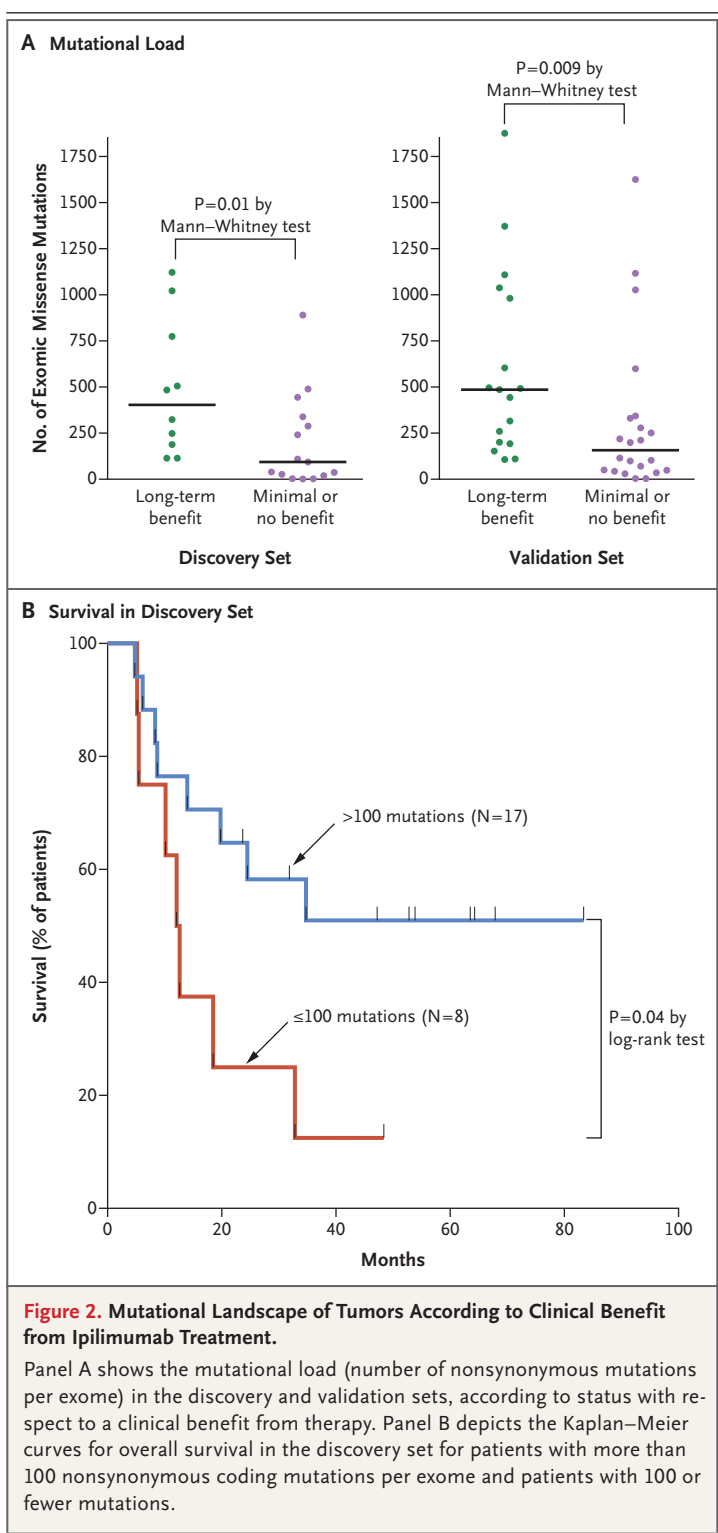### SOMATIC NEOEPITOPES IN RESPONDING TUMORS AND EFFICACY OF CTLA-4 BLOCKADE

MHC class I presentation and cytotoxic T-cell recognition are required for ipilimumab activity.[10] Because mutational load alone did not explain a clinical benefit from CTLA-4 blockade, we hypoth-

esized that the presence of specific tumor neoantigens might explain the varied therapeutic benefit. To identify these neoepitopes, we developed a bioinformatic pipeline incorporating prediction of MHC class I binding, modeling of T-cell receptor binding, patient-specific HLA type, and epitope-homology analysis (see the Methods section and Fig. S4 in the Supplementary Appendix).

We created a computational algorithm, called NAseek, to translate all nonsynonymous missense mutations into mutant and nonmutant peptides (see the Methods section and Fig. S4 in the Supplementary Appendix). We examined whether a subgroup of somatic neoepitopes would alter the strength of peptide–MHC binding, using patient-specific HLA types (Table S3 in the Supplementary Appendix). We first compared the overall antigenicity trend of all mutant versus nonmutant peptides. In aggregate, the mutant peptides were predicted to bind MHC class I molecules with higher affinity than the corresponding nonmutant peptides (Fig. S5 in the Supplementary Appendix).

Using only peptides predicted to bind to MHC class I molecules (binding affinity, ≤500 nM), we searched for conserved stretches of amino acids shared by multiple tumors. Using the methods described in the Methods section in the Supplementary Appendix, we identified shared, consensus sequences.[20] We identified a number of tetrapeptide sequences that were shared by patients with a long-term clinical benefit but completely absent in patients with a minimal benefit or no benefit (Fig. 3A and 3B, and Table S6 in the Supplementary Appendix). It has been shown that short amino acid substrings comprise conserved regions across antigens recognized by a T-cell receptor.[21] In these experiments, recognition of epitopes was driven by consensus tetrapeptides within the immunogenic peptides, and tetrapeptides within cross-reacting T-cell receptor epitopes were necessary and sufficient to drive T-cell proliferation, findings that are consistent with evidence that this polypeptide length can drive recognition by T-cell receptors.[22] Tetrapeptides are used to model genome phylogeny because they occur relatively infrequently in proteins and typically reflect function.[23]

We used the discovery set to generate a peptide signature from the candidate neoepitopes. This analysis initially pooled the aforementioned discovery and validation sets to remove low-frequency tetrapeptides in the combined exomes. Subsequent analysis is restricted to post-filtering peptides (see the Methods section in the Supple-

Figure 2. Mutational Landscape of Tumors According to Clinical Benefit from Ipilimumab Treatment.

Panel A shows the mutational load (number of nonsynonymous mutations per exome) in the discovery and validation sets, according to status with respect to a clinical benefit from therapy. Panel B depicts the Kaplan–Meier curves for overall survival in the discovery set for patients with more than 100 nonsynonymous coding mutations per exome and patients with 100 or fewer mutations.

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