Recurrent SERPINB3 and SERPINB4 mutations in patients who respond to anti-CTLA4 immunotherapy

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Immune checkpoint blockade has shown significant promise as an anticancer treatment, yet the determinants of response are not completely understood. Here we show that somatic mutations in SERPINB3 and SERPINB4 are associated with survival after anti-CTLA4 immunotherapy in two independent cohorts of patients with melanoma (n = 174). Interestingly, serpins are homologs of the well-known ovalbumin antigen and are associated with autoimmunity. Our findings have implications for the personalization of immunotherapy.

Immune checkpoint inhibitors have shown exceptional promise in the treatment of several advanced malignancies. For example, treatment with ipilimumab, an antibody to CTLA4, has increased survival rates for patients with melanoma1–3. Anti-PD1 blockade has shown therapeutic efficacy in cancers such as melanoma, non-small-cell lung cancer, and renal cell cancer, among others4–6. Understanding the genetic determinants of response to immune checkpoint blockade is critical for determining which patients will benefit from immunotherapy and for the design of more effective treatment options.

We and others have previously shown that the genetic features of cancers can shape the susceptibility of tumors to immune checkpoint blockade therapy4–9. For example, neoantigen load, mutational load, and tumor clonality can affect the likelihood of response to anti-CTLA4 or anti-PD1 therapy6–9. Previous sequencing studies have suggested that patients with lung cancer who have elevated levels of smoking-related mutagenesis are more likely to respond to anti-PD1 therapy7. However, it is unknown whether the presence of mutations in specific genes can influence response rates for immune checkpoint inhibitors in a manner analogous to that by which EGFR mutations predict response to erlotinib.

To address this issue in a rigorous manner, we analyzed the exomes of matched tumor and normal pairs from 174 patients with melanoma treated with anti-CTLA4 therapy. These patients were from two independent cohorts: one from the United States generated by us (n = 64; cohort 1) and a second from Germany (n = 110; cohort 2)6,9. These data, along with a recently published analysis of mutations in melanoma by The Cancer Genome Atlas (TCGA), now enable a gene-centric approach to detect recurrently mutated genes that predict survival11.

A comprehensive analysis of recurrent mutations in these data sets was performed to determine association with overall survival after anti-CTLA4 therapy (Online Methods and Supplementary Table 1)11. Strikingly, we discovered that SERPINB3 was recurrently mutated in patients deriving clinical benefit from anti-CTLA4 therapy. These mutations were strongly associated with overall survival following therapy in both independently collected cohorts (Fig. 1a). SERPINB3 encodes a serine protease inhibitor that functions in apoptosis and autoimmunity11–14. Interestingly, SERPINB3 is a human homolog of the chicken ovalbumin protein (OVA), which is a classic model antigen. We also found mutations in SERPINB4, a close human homolog of SERPINB3 with which it shares 92% protein sequence identity. SERPINB3 and SERPINB4 proteins have overlapping functions and are involved in both oncogenesis and immunity14–16.

We have therefore considered mutations in both genes together.

Mutations in either SERPINB3 or SERPINB4 were associated with significantly longer survival following anti-CTLA4 treatment in both cohorts (Fig. 1b). Notably, mutations in SERPINB3 and SERPINB4 did not associate with survival in patients with metastatic melanoma from TCGA, suggesting that these mutations are predictive of response to immunotherapy and are not simply prognostic (Fig. 1c). Patients with SERPINB3 or SERPINB4 mutations were also significantly more likely to experience clinical benefit from anti-CTLA4 therapy in both cohorts (Fig. 1d). Tumors with SERPINB3 or SERPINB4 mutations were by no means limited to highly mutated tumors (Fig. 1e), and multivariate analysis showed that mutations in these genes were associated with overall survival independent of mutation load (cohort 1, P = 0.05; cohort 2, P = 0.01; Online Methods and Supplementary Table 2). Mutations occurred in all four subtypes of melanoma (Fig. 2a). SERPINB3 and SERPINB4 mutations appeared to be mutually exclusive.

The characteristics and locations of these mutations are described in Figure 2b, Supplementary Figure 1, and Supplementary Table 3. The missense mutations that occur throughout both genes may alter protein activity and, in many cases, are predicted to produce immunogenic neopeptides (Supplementary Tables 4 and 5). Indeed, visualization of mutations on the solved 3D protein structures showed a cluster of variants near the active site of each protein, the reactive center loop (RCL) domain (Fig. 2c,d).

Because of the pleiotropic functions of serpin proteins, a mechanistic link between mutations in genes encoding serpins and immu-
notherapy response is likely multifaceted and complex. Although elucidation of these molecular details will require additional functional work, our genetic data may provide insight regarding which of the several aspects of serpin biology might be involved. SERPINB3 has been identified as a significantly and recurrently mutated gene in melanoma by TCGA and another group, pinpointing it as a driver of oncogenesis\textsuperscript{11,17}. Indeed, serpins are known to exhibit antiapoptotic functions\textsuperscript{13}, including suppression of UV-induced apoptosis in human

![Figure 1](image1.png)

**Figure 1** Somatic mutations of SERPINB3 and SERPINB4 predict improved survival from treatment with anti-CTLA4 therapy. (a) Overall survival of patients with SERPINB3 mutations in cohort 1 (\(n=64\)) and cohort 2 (\(n=110\)). WT, wild type; mut, mutant. (b) Overall survival of patients with either SERPINB3 or SERPINB4 mutations in cohort 1 and cohort 2. (c) Survival of patients with either SERPINB3 or SERPINB4 mutations exhibiting clinical benefit or response in cohort 1 and cohort 2. Statistical significance was evaluated by Fischer’s exact test; error bars, standard error. (d) Proportion of patients with SERPINB3 or SERPINB4 mutations as a function of mutation load. The middle line corresponds to the mean; error bars, s.e.m.

![Figure 2](image2.png)

**Figure 2** Characteristics of mutations in SERPINB3 and SERPINB4. (a) Oncoprints of SERPINB3 and SERPINB4 mutations in the two cohorts. (b) Diagrams showing the location of mutations in SERPINB3 and SERPINB4 (data from both cohorts combined). The blue segment for each protein represents the putative RCL domain. (c,d) Location of variants on the 3D protein structures of SERPINB3 (c) and SERPINB4 (d). Red, mutated amino acid; blue, putative RCL domain.
keratinocytes\textsuperscript{18}. There are also a number of possible mechanisms by which the observed SERPINB3 and SERPINB4 mutations may influence tumor immunogenicity. Mutations affecting various serpin family proteins are known to cause misfolding and self-polymerization, leading to the formation of inflammatory aggregates or plaques. These, in turn, function as targets in various autoimmune diseases, including systemic lupus erythematosus and psoriasis\textsuperscript{13,14,19,20}. Serpin polymers can also induce autophagy, thereby potentially entrapping autoantigen presentation\textsuperscript{13,21}. Therefore, mutant SERPINB3 and SERPINB4 proteins may act as a driver of melanoma tumorigenesis and/or an immunodeterminant, similar to mutant IDH1 in glioma\textsuperscript{22}.

SERPINB3 is a human homolog of the chicken egg protein OVA, a classic model antigen that contributes to egg allergies and atopic dermatitis in humans\textsuperscript{23}. OVA and SERPINB3 have sequence similarity, including over distinct regions functionally validated as epitopes of human OVA-reactive T cells\textsuperscript{24}. It has not escaped our notice that many of the observed SERPINB3 and SERPINB4 mutations map within these regions of homology (Supplementary Fig. 2). However, these epitopes may or may not serve as direct targets for the adaptive immune system throughout the course of metastatic disease, and alternative mechanisms such as cross-presentation and epitope spreading may be involved. Expression data from TCGA suggest that SERPINB3 and SERPINB4 proteins may exert an early immunogenic effect, thereby helping to initiate a broad immune response that can later be reinvigorated through checkpoint blockade. Additional mechanistic work will be required to clarify the role of SERPINB3 and SERPINB4 mutations in immunotherapy response. We believe our findings have broad implications for therapy response. We believe our findings have broad implications for therapy response. We believe our findings have broad implications for therapy response.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

T.A.C. and N.R. designed and conceived the study. Analysis of mutations in individual genes with outcome was performed by S.M.K., N.W., V.M., and J.J.H. Neoantigen analysis was performed by J.J.H., S.M.K., and V.M. Analysis of expression data was performed by L.A.W., A.D., and N.R. N.R., J.J.H., and T.A.C. prepared the manuscript. All authors participated in discussion of the final manuscript and interpretation of results.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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URLs. R project, http://www.r-project.org/; Immune Epitope Database (IEDB), http://www.iedb.org/.

Code availability. Code and ancillary data necessary to reproduce results and figures are available upon request.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Exome sequencing data are available in the database of Genotypes and Phenotypes (dbGaP): cohort 1, phs001041.v1.p1; cohort 2, phs000452.v2.p1.

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ONLINE METHODS

Mutational analysis and whole-exome sequencing. The patient cohorts treated with anti-CTLA4 therapy have been described previously. Whole-exome sequencing for cohort 1 and cohort 2 was performed with a minimum depth of coverage of 103-fold and 183.7-fold, respectively. Analysis was performed as previously described by DePristo et al. Briefly, paired-end reads in FASTQ format were aligned to the reference human genome GRCh37 using Burrows–Wheeler aligner (BWA; v0.7.10). Subsequently, local realignment was performed using the Genome Analysis Toolkit (GATK) version 3.2.2 (ref. 27). Duplicate reads were removed using Picard version 1.119. Somatic single-nucleotide variants (SNVs) were identified using a combination of four mutation callers, namely MuTect 1.1.4, Varscan 2.3.7, Somatic Sniper 1.0.4, and Strelka 2.2.6.2. SNVs with an allele read count of less than 5 or with normal coverage of less than 7 were removed. Small indels were called using GATK 3.2.2.

Statistics and survival analysis. Overall survival information and classification of patients into those with durable clinical benefit and those with minimal benefit were obtained from the original publications. Survival analysis was performed using the Kaplan–Meier method, with differences in survival determined by log-rank test. Multivariate survival analysis was performed using a Cox proportional hazards model. Differences in the distribution of quantitative variables between groups were determined by Wilcoxon rank-sum test, unless otherwise indicated. Differences in proportions between groups were determined by Fisher’s exact test, unless otherwise indicated. All statistical analyses were performed in the R statistical environment (v3.2).

Association of recurrent mutations with survival. Since our initial report on anti-CTLA4 therapy in melanoma, TCGA published a comprehensive genomic analysis of melanoma. We analyzed the 19 recurrently mutated genes in melanoma identified by InVex and described by TCGA (Supplementary Table 1). All 19 genes were tested for association with overall survival using the χ² test statistic from the ‘survival’ package in R and a permutation procedure. The overall procedure follows the concept described by Kim et al. We created n = 10,000 permutations of the binary mutation matrix (genes × samples), keeping row and column sums constant, thereby accounting for potential confounding factors such as mutation load. In each iteration, we recorded the χ² test statistic for association between permuted mutations and overall survival for all genes. An empirical P value was derived for each recurrently mutated gene by comparing observed test statistics to the distribution of simulated test statistics. Association of SERPINB3 with overall survival was significant after Bonferroni correction for multiple testing at P = 0.037 (uncorrected P = 0.005). No other genes were significant.

We subsequently verified that SERPINB3 was also associated with overall survival in an independently collected group of patients, cohort 2 (from Germany) (P = 0.05; Fig. 1a). As SERPINB3 and SERPINB4 are close homologs, we grouped mutations in these genes together. Multivariate analysis correcting for M stage and mutation load demonstrated that SERPINB3 and SERPINB4 mutations were associated with overall survival in cohort 1 (hazard ratio (HR) = 0.34, 95% confidence interval (CI) = 0.11–0.76, P = 0.05) and cohort 2 (HR = 0.32, 95% CI = 0.13–0.76, P = 0.01) (Supplementary Table 2).

Alignment of ovalbumin and SERPINB3. Clustal Omega was used to align OVA and SERPINB3. Epitopes presented for SERPINB3 were determined from the literature. Immunogenic epitopes from OVA were also determined from the literature. The Immune Epitope Database (IEDB) was accessed on 21 March 2016 and was used to identify all relevant epitopes.

Computational neoantigen prediction. Class I human leukocyte antigen (HLA) typing was performed manually for cohort 1 and was computed with Polysolver for cohort 2 from the exome data provided by the original authors. Each nonsynonymous SNV was translated into a 17-mer peptide sequence centered on the mutated amino acid. This 17-mer was then used to create 9-mers via a sliding window approach for determination of major histocompatibility complex (MHC) class I binding. netMHC version 3.4 was used to determine the binding strength of mutated peptides to patient-specific HLA alleles. All peptides with binding score IC₅₀ < 500 nM were considered to be putative MHC class I neoantigens. For MHC class II antigens, a 29-mer peptide sequence was created and a 15-mer sliding window approach was used. Class II HLA typing was performed with SOAP-HLA on both cohorts from exome data. netMHCpan version 3.1 was used to determine the affinity of mutated peptides for patient-specific HLA alleles, and peptides with a rank less than 2% were considered putative MHC class II neoantigens.