Tumor mutational burden correlates with response to immune checkpoint blockade in multiple solid tumors, although in microsatellite-stable tumors this association is of uncertain clinical utility. Here we uniformly analyzed whole-exome sequencing (WES) of 249 tumors and matched normal tissue from patients with clinically annotated outcomes to immune checkpoint therapy, including radiographic response, across multiple cancer types to examine additional tumor genomic features that contribute to selective response. Our analyses identified genomic correlates of response beyond mutational burden, including somatic events in individual driver genes, certain global mutational signatures, and specific HLA-restricted neoantigens. However, these features were often interrelated, highlighting the complexity of identifying genetic driver events that generate an immunoresponsive tumor environment. This study lays a path forward in analyzing large clinical cohorts in an integrated and multifaceted manner to enhance the ability to discover clinically meaningful predictive features of response to immune checkpoint blockade.
power to detect significant associations between pre-treatment tumor characteristics and response to immune checkpoint therapies. Thus, we gathered raw tumor and germline pre-treatment WES data for tumors from immune-checkpoint-therapy-treated patients in seven published studies (n = 171)\(^{7,8,10,22–25}\) and combined them with data from 78 newly sequenced pre-treatment tumors. By harmonizing clinical annotations and whole-exome analyses across the 249 samples and multiple cancer types, we aimed to (i) assess the generalizability of prior hypotheses regarding response to immune checkpoint blockade to other histological or drug settings; (ii) apply new computational techniques for inference of tumor biology and immunogenicity; and (iii) determine whether our cohort was of

Exclusions (n = 65):
- Clinical benefit unclear
- Tumor-in-normal contamination (>1%)
- Tumor sample contamination (>10%)
- Low tumor purity (<10%)
- Low normal mean target coverage (<15×)
- Low tumor mean target coverage (<25×)
- Failed sequencing realignment

n = 249 tumors in final analysis

Fig. 1 | Clinical cohort consolidation, response stratification, and mutational load investigation. a, Data quality control for the 249 samples included in the final analysis. b, Comparison of three published response metrics for determining objective response (OR) versus no response (NR). c, Comparison of tumor mutational burden between patients with complete response (CR) or partial response (PR) and those with progressive disease (PD) (for ‘All mutations’, P = 0.0005 for CR/PR versus PD, P = 0.0054 for CR/PR versus stable disease (SD), and P = 0.434 for SD versus PD; for ‘Nonsynonymous mutations’, P = 0.0003 for CR/PR versus PD, P = 0.0063 for CR/PR versus SD, and P = 0.38 for SD versus PD; for ‘Clonal nonsynonymous mutations’, P = 0.00005 for CR/PR versus PD, P = 0.011 for CR/PR versus SD, and P = 0.15 for SD versus PD). Outlying points from patients with >101 mutations/Mb are not shown (2 CR/PR, 1 SD, 3 PD). d, Intratumoral heterogeneity across response groups (n = 249 biologically independent samples; P = 0.001 for CR/PR versus PD, P = 0.51 for CR/PR versus SD). e, Clinical response to immune checkpoint therapy broken down by intratumoral heterogeneity. For c and d, P values were calculated by two-sided Mann–Whitney U test: *P < 0.05, **P < 0.005; ns, not significant. Box plots show the median, first and third quartiles, whiskers extend to 1.5 times the interquartile range, and outlying points are plotted individually.
sufficient size to identify statistically robust associations for specific genetic mediators of selective response.

Results
Consolidation of a clinically annotated cohort of whole-exome sequencing for tumors from patients treated with immune checkpoint blockade. WES from clinically annotated tumor samples with matched germline blood or adjacent normal tissue was available for 314 patients (Fig. 1a and Supplementary Table 1). Standard quality-control measures were taken to ensure adequate power to detect tumor-specific mutations (Fig. 1a, Methods, and Supplementary Fig. 1a–c). Our final analysis cohort included 249 patient tumors across six cancer types: melanoma (n = 151), non-small-cell lung cancer (NSCLC) (n = 56), small cell lung cancer (n = 1), bladder cancer (n = 27), head and neck squamous cell carcinoma (HNSCC) (n = 12), anal cancer (n = 1), and sarcoma (n = 1) (Fig. 1a). These patients were treated with anti-PD-1 (n = 74), anti-PD-L1 (n = 20), anti-CTLA-4 (n = 145), or a combination of anti-CTLA-4 and either anti-PD-1 or anti-PD-L1 therapies (n = 10). A small minority of patients received anti-PD-1, anti-PD-L1, or anti-CTLA-4 therapy in combination with another immunotherapy, targeted therapy, or cytotoxic chemotherapy (n = 7) (Supplementary Table 2). Across these 249 samples, the average mean target sequencing coverage was 150-fold for tumor tissue and 119-fold for matched germline tissue. The mean estimated tumor purity was 58% (range, 10–97%) (Supplementary Table 1).

In selecting a framework to assess clinical response in this study, we encountered multiple patient stratification methods in previous studies of response predictors to immune checkpoint therapy. These methods varied mostly in their treatment of patients with stable disease (SD) by RECIST 1.1, who have minimal to no change in tumor burden following therapy. Applying three existing response definitions to our cohort, we observed substantial differences in patient classification into responder and non-responder groups (Fig. 1b and Supplementary Table 3). Given the evolving viewpoints on classifying response to immune checkpoint blockade, we adopted a conservative method of defining objective response (OR) as complete response (CR) or partial response (PR) by RECIST (major decrease in tumor burden following treatment) and no response (NR) as progressive disease (PD) by RECIST (major increase in tumor burden following treatment) for the main analyses. Patients with stable disease were considered separately, and analyses using two other response definitions,11,12,16 that stratify patients with stable disease into those with objective response versus no response by duration of overall survival (OS) or progression-free survival (PFS) are available in the Supplementary Information.

Mutational burden and response to immune checkpoint therapy.
In examining whole-exome genetic features in this cohort, we began with tumor mutational burden, as this has been the most widely reproduced association with response to immune checkpoint therapy. We found that, in this combined cohort, patients with complete or partial response had significantly higher tumor mutational burdens than patients with progressive disease (P < 0.05 for all, Mann–Whitney U test) (Fig. 1c). This finding persisted within cancer types (Supplementary Fig. 2) and was particularly prominent in patients treated with PD-1/PD-L1 inhibitors (Supplementary Fig. 3a). Patients with stable disease tended to have mutational burdens intermediate between those of patients with progressive disease and those with complete or partial response, with higher mutational loads in patients with stable disease with long compared to short duration of overall survival (Supplementary Fig. 3b).

While these findings are consistent with the growing body of literature supporting the association between mutational burden and immune checkpoint therapy response, we noted that the ranges of the mutational burdens for the response groups overlapped considerably (Fig. 1c), and we found that tumor mutational burden had poor predictive power to differentiate complete or partial response from progressive disease as a single variable in this cohort (area under the receiver operator characteristic (ROC) curve (AUC) = 0.66) (Supplementary Fig. 4). In attempts to build upon the utility of tumor mutational burden as a predictive variable for response to immune checkpoint therapy, past studies have determined that clonal mutations (found in every cancer cell) rather than subclonal mutations (found in a subset of cancer cells) are more strongly associated with response in lung adenocarcinomas and some melanomas, potentially owing to stronger T cell responses to neoantigens generated from clonal versus subclonal mutations.41

We queried our cohort for this association by using absolute to infer mutational clonality (Methods) and demonstrated that clonal nonsynonymous mutational burden strongly predicted complete or partial response versus progressive disease across cancer types and response categorizations (Fig. 1c,d and Supplementary Fig. 2). Patients with a large proportion of subclonal mutations (>50%), which we term high intratumoral heterogeneity, were substantially more likely to have progressive disease than complete or partial response across all tumors described here (P = 0.0014, Fisher's exact test) (Fig. 1c). Thus, while mutational burden begins to explain the variance in patient response to immune checkpoint therapies, intratumoral heterogeneity contributes additional biological insight.

Mutations in specific genes associated with response or resistance to immune checkpoint therapy. Given the complexity we observed in the association between mutational load and response to immune checkpoint therapy, we pursued additional analyses of exome-level features that could provide further nuance to this association. We next investigated whether somatic mutations in specific genes were associated with response to immune checkpoint therapy, hypothesizing that this analysis would identify genes driving biological processes generating large numbers of mutations or those creating an immunoresponsive phenotype independently of mutational burden non-clinically annotated NSCLC exomes.

Given that response rates were similar across all cancer types (Supplementary Table 3), we first compared nonsynonymous mutations in patients with complete or partial response to those in patients with progressive disease across all genes; however, the associations found were too weak to pass correction for multiple-hypothesis testing (Supplementary Fig. 5). Thus, we limited our analysis to known hotspot mutations in cancer driver genes and to loss-of-function alterations in known tumor suppressors (Methods and Supplementary Table 4), as these events are more likely to have a significant impact on tumor biology. Clonal driver alterations in PIK3CA, KRAS, and PBRM1 were enriched in patients with complete or partial response, while clonal driver mutations in EGFR were enriched in patients with progressive disease (two-tailed Fisher's exact test, P < 0.05 for all genes; Benjamini–Hochberg false discovery rate (FDR) q = 0.18 for KRAS and PIK3CA) (Fig. 2a).

After correcting for tumor mutational burden, KRAS and PIK3CA remained associated with complete or partial response (P < 0.05, logistic regression) (Fig. 2b), although these observations did not pass FDR correction and may be confounded by additional aspects of a tumor's genetic profile.

Driver mutations within a given gene may occur in different functional domains and have different phenotypic effects, often depending on the cancer context. Thus, we next examined these cohort-wide gene associations for trends within specific cancer types. Of the patients with clonal hotspot mutations in PIK3CA, those with complete or partial response had melanoma, HNSCC, anal cancer, or bladder cancer, whereas the majority of those with stable or progressive disease had lung cancer (Fig. 2c, Supplementary Fig. 6, and Supplementary Table 5). Hotspot
mutations in KRAS occurred predominantly in patients with complete or partial response across multiple cancer types (Fig. 2d). Genetic events in PIK3CA and KRAS were too infrequent to fully clarify their relationships to response in this study, but these results demonstrate that single-gene associations with response to immune checkpoint therapy can provide additional information beyond mutational burden, in a manner that may be dependent on or independent of cancer type.

Despite combining data from multiple studies and cancer types, our analyses were still statistically underpowered to detect important relationships; thus, we sought to estimate the sample sizes needed for discovery of single-gene correlates of response with appropriate correction for multiple-hypothesis testing (Methods). We modeled statistical significance values for common or rare variants associated with complete or partial response versus progressive disease at various sample sizes (Fig. 2e). In the best-case scenario, where a variant is both relatively common (~10% prevalence) and specific to responders, sample sizes of around 300 would be adequate to detect significant associations. Meanwhile, detection of rare response-associated variants (~1% frequency), even if highly specific, would
necessitate sample sizes in the thousands. Thus, continuing to combine independent clinically annotated cohorts will aid in increasing statistical power to detect common response-associated variants, but for detection of rarer events, applying insights from experimental studies for hypothesis-driven validation will be a crucial adjunct.

Integrated analysis of response- and resistance-associated mutations with mutational signatures. As a further step toward understanding the mutational processes that generate an immunoresponsive tumor environment, we next investigated whole-exome signatures of mutagenic biological processes. We used a previously described non-negative matrix factorization technique to identify known mutagenic processes in lung cancer, melanoma, bladder cancer, and HNSCC\textsuperscript{26,29} (Methods). Using this technique, the somatic mutations within a tumor are probabilistically assigned to underlying mutational signatures, which are patterns of somatic mutations thought to arise from carcinogenic processes (for example, a predominance of C-to-A transversions in tobacco-smoking-associated cancers or C-to-T transitions in UV-light-associated tumors) (Supplementary Tables 6 and 7). As has been seen previously in analyses of non–clinically annotated NSCLC exomes\textsuperscript{30}, pre-treatment lung tumors in this cohort with a high proportion of smoking-associated mutations tended to have low intratumoral heterogeneity, high mutational burden, and frequent KRAS mutations (Fig. 3a–c). The proportion of smoking-associated mutations was also higher in patients with complete or partial response than in those with progressive disease (Supplementary Fig. 7a). Meanwhile, tumors with \textit{EGFR} hotspot mutations tended toward enrichment in subclonal mutations (\(P = 0.035\)), had low mutational burdens, were over-represented in never-smokers (two-tailed Fisher’s exact test, \(P = 0.00017\)), and had generally poor responses to immune checkpoint therapy (Fig. 3a,d), which is also consistent with previously published results\textsuperscript{1,2,5}. Thus, the association between KRAS and \textit{EGFR} mutation status and response to immunotherapy (Fig. 2a,b) may be related not only to mutational burden but also to mutational signature and mutational clonality. After controlling for smoking history, mutational burden remained a significant predictor of response (Fig. 3e and Supplementary Fig. 7b). Concurrent consideration of mutational signatures, clonal architecture, and hotspot mutations in NSCLC enhances understanding of somatic mediators of immunotherapy response and resistance.

We also examined mutational signatures in melanoma tumors, which are dominated by exposure to UV light (S7), prior chemotherapeutic treatment with alkylating agents (S11), and other signatures not clearly associated with specific environmental exposures (S1 and S5). Dominant mutational signature explained a large proportion of the variance in mutational burden and was highly correlated with intratumoral heterogeneity (Fig. 4a and Supplementary Fig. 8a). After stratifying by dominant mutational signature, no significant difference in mutational burden was observed between patients with complete or partial response and those with progressive disease (\(P > 0.05\) for all) (Fig. 4b). The non-UV/non-alkylating group had a higher proportion of patients with progressive disease than the other two groups (Fig. 4c) and was composed largely of mucosal, uveal, and acral lentiginous melanomas, although dominant mutational signature and histology did not overlap perfectly (Supplementary Fig. 8b). The observation that mutational load is not a significant predictor of response after correcting for dominant mutational signature in melanoma raises the possibility that, in this cancer type, mutational burden itself may not directly mediate response but rather may serve as a proxy for an underlying biological process that both increases tumor immunogenicity and promotes accumulation of somatic mutations.

In bladder cancer and HNSCC, similar analyses demonstrated association of APOBEC-associated signatures (S2 and S13) with higher mutational burdens (\(P = 0.002\) for bladder cancer, \(P = 0.03\) for HNSCC, two-sided Mann–Whitney \(U\) test) and greater likelihood of complete or partial response (\(P = 0.019\)) (Fig. 4d–g). APOBEC signatures have been linked with tobacco exposure\textsuperscript{11}, but viral infection and \textit{PIK3CA} hotspot mutations are other potential etiologies\textsuperscript{11}. These correlations may contribute to and/or confound the observed association between \textit{PIK3CA} hotspot mutations and complete or partial response described above (Fig. 2c and Supplementary Fig. 6). APOBEC mRNA expression has also previously been associated with increased PD-L1 immunohistochemical staining\textsuperscript{23} and high tumor mutational burden\textsuperscript{26} in urothelial carcinoma. Overall, genetic features—including mutational burden, intratumoral heterogeneity, tumor driver mutations, and mutational signatures—appear to have interrelated associations with response in this cohort. Determining which of these features ultimately drives response to immune checkpoint therapy in these patients will require further clinical and experimental study.

Copy number alterations associated with response or resistance to immune checkpoint therapy. In addition to somatic mutations, CNAs may also contribute to selective response, and CNAs affecting the interferon-\(\gamma\) pathway have been implicated in intrinsic resistance to immune checkpoint therapies in melanoma\textsuperscript{2}. In this cohort, we assessed CNAs expected to interfere with interferon-\(\gamma\) signaling after correcting for tumor purity (Methods), which can strongly influence the number of called CNAs in cohorts with heterogeneous tumor purity and ploidy (Supplementary Fig. 9a–c). After correcting for tumor purity, interferon-\(\gamma\)-related CNA events were more infrequent than previously described, but consistent with prior studies these events were enriched in patients with progressive disease in comparison to those with complete or partial response (19/123 versus 3/70; \(P = 0.019\), Fisher’s exact test) (Fig. 5a). This relationship persisted within cancer types and therapy classes, although this study was insufficiently powered to detect a significant association in many of the subgroup analyses (Fig. 5b,c and Supplementary Figs. 10 and 11).

Next, we analyzed focal CNAs affecting 63 commonly amplified and deleted tumor suppressors and oncogenes (Methods and Supplementary Tables 8 and 9) to nominate additional mediators of selective response or resistance. While these events were rare and no gene achieved statistical significance alone, amplifications of \textit{PAK1}, \textit{YAP1}, and \textit{CCND1} on chromosome 11q and amplifications of \textit{MDM2} and \textit{CDK4} on chromosome 12q were seen predominantly in patients with progressive disease (Fig. 5d), with the latter of note as CDK4/CDK6 inhibition was recently associated with increased tumor immunogenicity\textsuperscript{7}. Additionally, homozygous \textit{PTEN} deletion occurred exclusively in patients with intrinsic resistance to immune checkpoint therapy (\(n = 4\) with progressive disease; \(P = 0.30\)) (Fig. 5d), recapitulating the observation of biallelic \textit{PTEN} loss in resistance to immunotherapies in prior clinical and experimental studies\textsuperscript{12,19}. Clonal biallelic loss of \textit{PTEN} via truncating mutation was not as clearly associated with progressive disease, although many of these events were splice-site mutations of uncertain biological significance (Fig. 5e). Truncating \textit{PTEN} mutations were also seen in resistant tumors from patients with tumor shrinkage following anti-CTLA-4 therapy at other sites, although these patients were excluded from the main analysis owing to their mixed clinical response (Supplementary Fig. 12 and Supplementary Table 1). Thus, both mutations and CNAs can influence response to immune checkpoint therapy by activating or suppressing pathways that interact with tumor–immune signaling, although our analyses indicate that these features are elaborately interrelated and require substantially increased cohort size and mechanistic validation for robust interpretation.

Validation of previously described response predictors for immune checkpoint therapies. Previous studies have identified additional pathways and genes associated with response or resistance
Fig. 3 | Integrated analysis of EGFR mutational status, intratumoral heterogeneity, and mutational signatures in lung cancer. a, Stacked plots show mutational burden (histogram, top), estimated tumor purity (tile plot, top), mutations in EGFR (tile plot, middle), mutational signatures (filled histogram, middle), and clinical response and clinical covariates (bottom). b, Interaction between smoking-related mutational signatures and mutational burden. For aging/unknown versus smoking signatures, \( P = 0.0001 \); for aging/unknown versus APOBEC, \( P = 0.43 \); for APOBEC versus smoking, \( P = 0.158 \). c, Proportion of patients with a given dominant mutational signature by clonal mutation composition. d, Proportion of subclonal mutations in EGFR-mutant versus EGFR-wild-type tumors (\( n = 57 \) biologically independent samples, \( P = 0.035 \), unpaired two-sample \( t \) test). e, Relationship between mutational burden and response by dominant mutational signature (aging/unknown-dominant samples: CR/PR versus PD, \( P = 0.0044 \); CR/PR versus SD, \( P = 0.07 \); SD versus PD, \( P = 0.060 \); APOBEC/smoking-dominant samples: CR/PR versus PD, \( P = 0.0023 \); CR/PR versus SD, \( P = 0.08 \); SD versus PD, \( P = 0.25 \)). A two-sided Mann-Whitney \( U \) test was used in b and e: * \( P < 0.05 \), ** \( P < 0.005 \); ns, not significant. The box plots in b, d and e show the median, first and third quartiles, whiskers extend to 1.5 times the interquartile range, and outlying points are plotted individually.
Fig. 4 | Integrated analysis of mutational burden, intratumoral heterogeneity, and mutational signatures in melanoma, HNSCC, and bladder cancer. 

**a.** Nonsynonymous mutational burden (alkylating versus UV dominant, \( P = 0.0005 \); UV versus other dominant, \( P = 6.079 \times 10^{-14} \)) and clonal mutational burden (alkylating versus UV dominant, \( P = 0.938 \), UV versus other dominant, \( P = 6.404 \times 10^{-15} \)) stratified by dominant mutational signature in only patients with melanoma. 

**b.** Association between mutational burden and response within dominant mutational signatures in melanoma (CR/PR versus PD in alkylating dominant, \( P = 0.2 \); CR/PR versus PD in UV dominant, \( P = 0.549 \); CR/PR versus PD in other dominant, \( P = 0.689 \)). 

**c.** Likelihood of response by dominant mutational signature group in melanoma. 

**d, e.** Stacked plots show mutational burden (histogram, top), tumor purity (tile plot, top), mutational signatures (filled histogram, middle), and histology and clinical response to immune checkpoint therapy (tile plots, bottom) for HNSCC (d) and bladder cancer (e). 

**f.** Proportion of mutations attributable to APOBEC mutational signatures (S2 or S13) versus nonsynonymous mutational burden (\( n = 39 \) biologically independent samples; \( P = 2.66 \times 10^{-6} \) for slope, \( P = 0.848 \) for intercept). Symbols indicate cancer type. 

**g.** Proportion of mutations probabilistically attributable to the APOBEC mutational signature in CR/PR versus PD in HNSCC and bladder cancer (\( P = 0.019 \), \( n = 39 \)). A two-sided Mann–Whitney \( U \) test was used for all comparisons: \( * P < 0.05 \); \( ** P < 0.005 \); ns, not significant. The box plots in **a, b** and **g** show the median, first and third quartiles, whiskers extend to 1.5 times the interquartile range, and outlying points are plotted individually.
to immune checkpoint therapy, which were not identified via the unbiased analyses presented thus far, potentially owing to insufficient power after correcting for multiple-hypothesis testing. We attempted a focused validation of the previous findings in this cohort, beginning with an analysis of loss-of-function alterations in \( PBRM1 \), a member of the PBAF form of the SWI/SNF complex. Prior work in clear-cell renal cell carcinoma (ccRCC), a cancer type not represented in this study, demonstrated that biallelic \( PBRM1 \) loss correlates with response to anti-PD-1or anti-PD-L1 therapy, while mutational load does not\(^{19} \). Additional functional data in melanoma have supported this association and implicated other related genes in the SWI/SNF family of chromatin remodelers\(^{39} \).

In this cohort, truncating mutations in \( PBRM1 \) and other related epigenetic regulators in the SWI/SNF complex were also over-represented in responders, with biallelic loss events occurring almost exclusively in the tumors from patients with complete or partial response.

**Fig. 5** Tumor copy number alterations associated with response to immune checkpoint therapy. a–c, Amplifications and deletions of genes in the interferon-\( \gamma \) signaling pathway in CR/PR versus PD across all samples (two-tailed Fisher’s exact test \( P = 0.019 \), 95% confidence interval (CI) = 0.05–0.885, \( n = 193 \)) (a), samples classified by drug class (\( n = 120 \) anti-CTLA-4 treated and 65 anti-PD-1/PD-L1 treated) (b), and samples classified by cancer type (\( n = 23 \) bladder cancer, 7 HSNCC, and 125 melanoma) (c). Data are shown as the proportion of CR/PR or PD samples with CNAs ± standard error (\( ^*P < 0.05 \)). d, Differences in the proportion of CR/PR versus PD samples harboring focal cancer driver CNAs. The \(-\log_{10} P \) values from two-tailed Fisher’s exact tests for enrichment of a gene-level CNA in CR/PR versus PD are shown on the y axis. Genes more commonly affected by CNAs in CR/PR samples are shown on the right, while those more commonly deleted or amplified in PD samples are shown on the left. The dashed red line indicates \( P = 0.05 \) (\( n = 193 \) biologically independent samples). e, Truncating mutations in PTEN by response group. f, Prevalence and response association of truncating alterations in genes encoding SWI/SNF subunits\(^{45} \). Dark blue tiles indicate membership in either PBAF or BAF, which are complexes within the SWI/SNF family. Only genes encoding SWI/SNF subunits harboring truncating mutations in at least two patients are shown.
Recent studies have demonstrated that personalized cancer vaccines targeting neoantigens specific to an individual’s tumor can lead to durable clinical benefit alone or in concert with immune checkpoint blockade therapies. More than 99% of the predicted neoantigens in this study arose from passenger mutations, which occur throughout the exome, are frequently found in subclonal tumor populations, and are largely unique to each patient’s tumor. However, 871 predicted neoantigens were generated by driver mutations, and 8 of these ‘driver’ neoantigens occurred recurrently in patients with complete or partial response but not in patients with progressive disease, in a human leukocyte antigen (HLA)-dependent manner (Fig. 6b,c). Additionally, as expected given the known oncogenic effects of the mutations yielding these neoantigens, these eight neoantigens were clonal in all samples, suggesting that a T cell–mediated response, if present, would target all cancer cells. Thus, driver alterations can generate tumor neoantigens and may contribute to provoking an effective immune response to checkpoint blockade therapy in HLA-matched patients, although further experimental study is required to clarify the biological significance of these putative neoantigens.

**Discussion**

WES and analysis of 249 tumors from patients treated with anti-PD-1/PD-L1 or anti-CTLA-4 therapies suggest that genomic features beyond mutational burden, including genetic driver events, intratumoral heterogeneity, and mutational signatures, may affect response to immune checkpoint blockade. In this work, we combined data from multiple institutions using a standardized computational pipeline and applied a uniform and well-accepted definition of radiographic response to cancer therapy to more robustly assess genetic predictors of response to immune checkpoint therapy.
In so doing, we validated past findings and expanded their generalizability to new cancer contexts, discovered new correlative biomarkers of response using a larger sample size with more statistical power, and investigated the relationships between predictive biomarkers in enhanced detail. For instance, biallelic PTEN loss was first clinically described in acquired resistance to anti-PD-1 therapy in metastatic uterine leiomyosarcoma, and these clinical results and previous preclinical findings suggest that it may be relevant to intrinsic resistance and mixed response in metastatic melanoma as well\(^\text{37,38}\). CNAs leading to loss of intact interferon-γ signaling were previously noted in metastatic melanoma intrinsically resistant to treatment with anti-CTLA-4 agents\(^\text{40}\), but this mechanism may also have relevance to anti-PD-1/PD-L1-treated patients and in additional cancer types. PBRM1 loss was first implicated in increasing tumor intrinsic responsiveness to immune checkpoint therapy in ccRCC\(^\text{7,31}\), but shared biology from loss of PBRM1 or ARID2—which both encode proteins within the PBAF form of the SWI/SNF complex—in melanoma, lung cancer, bladder cancer, and HNSCC may underlie the similar response association observed here. Notably, experimental descriptions of the mechanistic underpinnings of these genetic variants in influencing response or resistance to T cell–mediated killing have been instrumental in supporting computational results\(^\text{19,20}\), emphasizing the importance of cross-validation of clinically relevant tumor variants in mechanistically driven investigations.

Such findings show that comprehensive consideration of multiple genomic features may help place existing associations such as mutational burden in a broader biological context. In the melanoma tumors from this study, mutational burden was no longer a significant predictor of response after correcting for dominant mutational signature, a finding that warrants further experimental and translational inquiry. KRAS and EGFR mutations in lung cancer have previously been described as being associated with response and resistance, respectively; this study demonstrates a relationship between these driver mutations and carcinoenic exposures, intratumoral heterogeneity, and mutational burden\(^\text{21,22}\). The global approach outlined herein will be essential in future investigations of predictors of immune checkpoint therapy response.

Power calculations suggest that combining hundreds or even thousands of clinically annotated patient samples will be necessary to reliably detect specific predictors of response to immune checkpoint therapies. While we preliminarily assessed response predictors specific to anti-CTLA-4 versus anti-PD-1/PD-L1 therapies or within a given cancer type, these findings are biased by the data available from clinically annotated cohorts; anti-CTLA-4 therapies were used predominantly in melanoma and anti-PD-1/PD-L1 therapies dominated most other cancer types. Further studies more directly comparing therapy classes within the same tumor histology, and vice versa, will be necessary, as will consideration of response predictors for combinations of checkpoint inhibitors with or without targeted or cytotoxic chemotherapies. Exceptions to single-feature genomic associations between this study and previous works likely can be explained by complex context-dependent effects and emphasize the need for caution to avoid over-interpretation of results.

While sample size and cohort heterogeneity remain major limitations of this work, this study describes a path forward for gathering insights from multiple clinically annotated patient cohorts. Our work advances hypotheses of biological mechanisms, suggests clinically relevant biomarkers, and highlights the importance of further, larger studies to reliably and robustly identify biomarkers of response and intrinsic resistance to immune checkpoint blockade.

**Methods**

Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41588-018-0200-2.
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**Author contributions**

D.M., C.A.M., N.I.V., A.T.-W., D.L., D.A., P.P., G.G., and D.K. performed the analyses. B.S., M.M., M.M.A., N.G.C., G.J.H., R.H., and S.M.W. provided clinical annotations. L.M.S., S.S., and S.I.R. contributed to immunohistochemical profiling. K.-K.W., J.A.E., M.M.A., D.A.B., R.I.H., D.S., F.S.H., T.K.C., J.B., P.A.J., R.H., A.T., P.H., and E.M.V.A. contributed to sample acquisition. E.M.V.A. supervised the study. D.M., C.A.M., N.I.V., D.L., and E.M.V.A. wrote the manuscript with contributions from all authors.

**Competing interests**

A.T., D.L., D.M., M.M., N.I.V., C.A.M., D.A., D.K., S.M.W., L.M.S., A.T.-W., P.P., K.-K.W., S.I.R., J.B., P.A.J., N.G.C., R.H., and M.M.A. declare no conflicts of interest. T.K.C. has advisory roles with AstraZeneca, Bayer, Bristol-Myers Squibb, Cereixel, Elsan, Foundation Medicine, Genentech, GlaxoSmithKline, Merck, Novartis, Pfizer, Prometheus Labs, Roche, and Eisai. T.K.C. receives research funding from AstraZeneca, Bristol-Myers Squibb, Exelixis, Genentech, GlaxoSmithKline, Merck, Novartis, Pfizer, Roche, Tracom, and Eisai. G.J.H. receives institutional support from Bristol-Myers Squibb and EMD Serono. B.S. is on the advisory board or has received honoraria from Novartis, Roche, Bristol-Myers Squibb, and MSD Sharp & Dohme, research funding from Bristol-Myers Squibb and MSD Sharp & Dohme, and travel support from Novartis, Roche, Bristol-Myers Squibb, and Amgen. R.I.H. has advisory roles with Bristol-Myers Squibb, Pfizer, Merck, AstraZeneca, Genentech, and Celgene. R.I.H. receives research funding from Bristol-Myers Squibb, Merck, Genentech, and Pfizer. S.S. is a consultant for AstraZeneca and Merck and receives research funding from AstraZeneca, Bristol-Myers Squibb, Exelixis, and Roche. G.G. has an advisory role with MD Anderson and receives research funding from IBM and Bayer. G.G. is listed as an inventor on patent applications regarding MuTect, ABSOLUTE, and Polysolver. D.A.B. is a consultant for N of One. D.S. receives consulting fees from AstraZeneca, GlaxoSmithKline, BMS, Novartis, Roche, Amgen, Merck, AstraZeneca, Merck-Serono, and Pfizer. P.H. and J.A.E. are employees of Novartis. E.S.H. is a consultant to Bristol-Myers Squibb, Merck, Novartis, EMD Serono, Sanofi, and Genentech and receives institutional research support from Bristol-Myers Squibb. E.M.V.A. holds consulting roles with Tango Therapeutics, Invitae, and Genome Medical and receives research support from Bristol-Myers Squibb and Novartis.

**Additional information**

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Methods
Clinical cohort consolidation. Patients from the Dana-Farber Cancer Institute with metastatic bladder cancer, HNSCC, lung cancer, or melanoma treated with anti- PD-1, anti-PD-L1, anti-CTLA-4, or a combination of these therapies were identified, and pretreatment tumor tissue and matched germline blood were obtained for genetic sequencing. These studies were conducted in accordance with the Declaration of Helsinki and approved by the Dana-Farber Cancer Institute Institutional Review Board (protocols 11-104, 05-042, 02-180, 09-472, 02-021, and 15-330). Electronic medical charts were reviewed to assess best response by RECIST (v1.1), duration of progression-free survival, duration of overall survival, patient demographic characteristics, and other relevant clinical details (for example, smoking history). 'Current/former' smokers were those who reported ≥ 5 pack-years (packs per day × years smoking) of tobacco use. 'Never' smokers were those who reported ≤ 5 pack-years. Clinical information from studies conducted outside the Dana-Farber Cancer Institute was downloaded from online supplementary information. Where overall survival was not reported in these studies, it was censored at progression-free survival.

Response stratification. Patients were divided into objective responder and non-responder groups according to three published response metrics, using best response by RECIST, duration of progression-free survival, and duration of overall survival. For patients in the MSKCC melanoma cohort, all non-responders were presumed to have progressive disease as their best response by RECIST. Tumor samples from progressing lesions from patients who had clinical benefit from immune checkpoint therapy were excluded from the main analysis. Tumors from patients with >50% progression-free survival or loss of clone duration of at least 30 d were excluded from analysis, as these patients may have had disease that was too advanced to experience clinical benefit from immune checkpoint therapy.

DNA extraction and sequencing. For samples newly sequenced from the Dana-Farber Cancer Institute, DNA extraction from formalin-fixed, paraffin-embedded (FFPE) tumor blocks was performed as previously described. Exome sequencing and data processing to produce a BAM file were performed using established analytical pipelines at the Broad Institute.

DNA sequencing quality control. Data from newly sequenced samples were combined with raw sequencing data (BAM files) from previously published cohorts of tumor–normal sequencing from patients with metastatic melanoma, lung cancer, anal cancer, and sarcoma. All 314 samples with tumor and germline sequencing data and clinical annotations were processed through standard quality-control pipelines. Samples with poor sequencing coverage (tumor mean target coverage < 25 ×, normal mean target coverage < 15 ×) or high sample contamination in tumor or normal tissue were excluded (Fig. 1a and Supplementary Table 1). Additionally, samples with germline sequencing data from adjacent normal tissue were assessed for tumor-in-normal contamination using deTin49 and excluded if the normal tissue contained ≥ 1% tumor nuclei (Fig. 1a and Supplementary Table 1). After mutation calling and somatic copy number variation assessment, tumors with estimated purity below 10% were also excluded (Supplementary Fig. 1a,b). These quality-control measures were taken because high sample contamination and low tumor purity can lead to systematic under-calling of somatic SNPs and CNAs and interference with accurate assessment of tumor mutational burden and identification of response-associated molecular features.

Whole-exome analysis. Somatic SNPs were identified by MuTect, with computational filtering of artifacts introduced by DNA oxidation during sequencing or FFPE-based DNA extraction using a filter-based method. Strelka2 was applied to detect small indels. Annotation of the variants identified was performed using Oncotator (https://software.broadinstitute.org/cancer/oncotator) and cga/oncotator. Mutational clonality was estimated by ABSOLUTE, which uses the allelic fraction of called mutations and allelic copy number information to determine mutational clonality and overall tumor purity and ploidy. Clonal mutations were defined as those with estimated cancer cell fraction (CCF) of 1 or those whose probability of being clonal exceeded the probability of being subclonal. Nonsynonymous mutational burden was normalized by megabases covered at adequate depth to detect variants with 80% power using MuTect given estimated tumor purity by ABSOLUTE (Supplementary Table 1). The number of bases covered at a given depth threshold in the tumor sample was determined using the GATK DepthOfCoverage module. Putative driver mutations were collected using cBioPortal standards, including both 3D hotspots in tumor suppressors and oncogenes and any loss-of-function variant in a tumor suppressor.

For copy number analysis, copy ratios were calculated for each captured target by dividing the tumor coverage by the median coverage obtained in a set of reference normal samples. The resulting copy ratios were segmented using the circular binary segmentation algorithm. Segments were considered amplified or deleted if the |log₂ (copy ratio)| exceeded 0.5 (Supplementary Fig. 9a). For samples with uniform sample purity and ploidy, this definition of amplifications and deletions is adequate to detect CNAs. However, in this sample of tumors, called CNAs were heavily influenced by sample purity (Supplementary Fig. 9a).

The |log₂ (copy ratio)| > 0.5 definition was insufficiently sensitive in low-purity samples, which have artificially depressed copy ratios owing to a high proportion of sequencing reads from normal tissue. Conversely, this definition may be excessively noisy in high-purity tumors.

Thus, to correct segment copy ratios for sample purity, segment copy ratios were rescaled by sample purity and ploidy with values derived from ABSOLUTE. Segments were considered amplified or deleted if the |log₂ (purity-corrected copy ratio)| exceeded 0.5. Specifically, the purity-corrected copy ratio was derived by dividing the purity-corrected total copy number (rCN) for a given segment by the sample ploidy. This procedure yielded improved false negative rates in low-purity tumors and false positive rates in high-purity tumors, such that the proportion of a tumor genome considered amplified or deleted was less closely associated with tumor purity (Supplementary Fig. 9b).

However, while using a |log₂ (purity-corrected copy ratio)| > 0.5 definition of deletions and amplifications was effective for detecting large CNAs with high sensitivity, it did not provide adequate specificity for detecting focal events that would be more likely to be genomic driver CNAs for a tumor. Thus, we applied a previously described concept called focality to identify CNAs that were large outliers in copy ratio, representing either homozygous deletions expected to completely eliminate tumor expression of a given gene or amplifications expected to greatly overexpress a gene. In this process, the rescaled copy number (rCN) from ABSOLUTE was used as input. For each segment in a tumor genome, the focality was calculated by considering the fraction of a sample’s genome with lower rCN than that segment (for amplified regions) or higher rCN (for deleted regions). Segments whose rCN was < 0.995 were considered amplified if their focality exceeded 0.98 – 0.2 × log₂(rCN/5) and highly amplified if their focality exceeded 0.98 – (1/7) × log₂(rCN/7). The results from this analysis are displayed in Supplementary Fig. 9c; far fewer segments met the criteria for being called amplifications or deletions under this focality-based definition. This focality-based definition of CNA was applied in Fig. 1f, Supplementary Figs. 12–13 to demonstrate that many CNAs in genes in the interferon-γ signaling pathway, PTEN, and genes in the SWI/SNF family of chromatin regulators. Genes were considered amplified or deleted if all or part of the gene was in a segment with a called CNA using this focality-based definition. For the interferon-γ analysis, samples were defined as having a CNA affecting interferon signaling if any of the regular intercellular signaling pathways were affected or any of the four interferon pathway inhibitors (SOCS1, SOCS3, PI4KA, and PI4S1) harbored high amplifications, as previously described. All gene-level CNAs of interest were manually reviewed.

A similar focality procedure was applied to allelic copy number calls from ABSOLUTE to detect heterozygous deletions and amplifications to identify loss-of-heterozygosity events (Supplementary Figs. 12 and 13).

Mutational signature deconvolution was conducted using a non-negative matrix factorization technique as previously described. Mutational signatures were chosen from those previously described in COSMIC (http://cancer.sanger.ac.uk/cosmic/signatures) (Supplementary Table 6). The vectors for the commonly observed mutational signatures for each cancer type were used as input for inference of their contribution to observed mutations. Thus, for example, the signatures selected for melanoma pertained to UV exposure, prior alkylating agents, and other exposures, while those used for mutational signature deconvolution in lung cancer included tobacco exposure.

In this pre-neoantigen prediction, the four-digit HLA type for each sample was inferred using Polysolver. Putative neoantigens were predicted for each patient by defining all novel amino acid 9-mers and 10-mers resulting from each somatic nonsynonymous point mutation and determining whether the predicted binding rank—a proxy for predicted binding affinity to the patient’s germline HLA alleles—was ≤ 2%. Strong binders had rank < 0.5%, while weak binders had rank between 0.5% and 2%, using NetMHCpan (v3.0)24–26.

Statistical analysis. Assessment of enrichment of binary molecular features (for example, wild-type or mutant gene; CNA present or absent) with response (complete or partial response versus progressive disease) was done with Fisher’s exact tests. Assessment of differences in means or medians for a continuous variable between two response groups was done with non-parametric Mann-Whitney U tests unless otherwise specified. Correction for multiple-hypothesis testing was done controlling for FDR by the Benjamini–Hochberg method unless otherwise noted. ROC analyses were done using the pROC and Epi packages in R. For the power calculation analysis (Fig. 2c), correction for multiple-hypothesis testing was modeled with a Bonferroni correction over the 116 genes with known cancer driver status assessed previously. Response rates were set at 40% complete or partial response versus 60% progressive disease, which is a generous estimate for response rate in an unselected population. P values were calculated using Fisher’s exact tests comparing the prevalence of mutations in a given gene in complete or partial response versus progressive disease.

The alpha level for all comparisons was 0.05 unless indicated otherwise. All statistical analyses were done in R (v.3.3.2).

Accession codes. Raw BAM files from previously published cohorts are available at the database of Genotypes and Phenotypes (dbGaP) under the following accession.
numbers: phs000694.v3.p2, phs000980.v1.p1, phs001041.v1.p1, phs000452.v2.p1, and phs001075.v1.p1.

**Reporting Summary.** Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** Newly sequenced samples are available at dbGaP under accession number phs001565.v1.p1.

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Experimental design

1. Sample size
   Describe how sample size was determined.
   Sample size was determined by systematic access and analysis of all available cases that met criteria, and represents the largest cohort assembled for this kind of analysis. Throughout the text we note when our power is limited by sample size, and we include a power simulation in Fig 2e where we determine that our sample size is adequate to detect a variant that is enriched in responders if the variant is present at 10% in the population.

2. Data exclusions
   Describe any data exclusions.
   We gathered whole exome sequencing from patients treated with immunotherapy in published and unpublished samples. Patients were excluded if they did not have reported response to immunotherapy or if they had PFS or OS < 30 days from start of therapy. We further excluded samples that failed sequencing re-alignment, or that failed whole exome sequencing quality control, excluding samples with ≥1% tumor in normal contamination, mean target coverage < 25x in tumor and < 15x in normal, tumor sample contamination ≥5%, and tumor purity < 10%.

3. Replication
   Describe whether the experimental findings were reliably reproduced.
   There were no experimental findings to reproduce.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.
   Our study analyzed genomic determinants of response to immunotherapy by stratifying samples into responders or non-responders. This was a retrospective analysis and as such, did not rely on prospective randomization of samples into experimental groups.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   As described above, this was a retrospective analysis of correlates of treatment response. Outcomes had already been determined for each sample prior to our analysis, and our subsequent analysis required assigning samples to response groups, making further blinding inappropriate.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.
6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

| n/a | Confirmed |
|-----|-----------|
| ☑️ | ☑️ | The exact sample size ($n$) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.) |
| ☑️ | ☑️ | A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
| ☑️ | ☑️ | A statement indicating how many times each experiment was replicated |
| ☑️ | ☑️ | The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section) |
| ☑️ | ☑️ | A description of any assumptions or corrections, such as an adjustment for multiple comparisons |
| ☑️ | ☑️ | The test results (e.g. $P$ values) given as exact values whenever possible and with confidence intervals noted |
| ☑️ | ☑️ | A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range) |
| ☑️ | ☑️ | Clearly defined error bars |

See the web collection on statistics for biologists for further resources and guidance.

Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

Single nucleotide variants were called with MuTect (version 1.1.6) and insertions/deletions were called with Strelka (version 1.0.11). Artifacts introduced by oxidation or FFPE-based DNA extraction were filtered according to previously published methods (Costello, M. et al, Nucleic Acids Res. 41, e67 (2013); Van Allen, E. M. et al. Nat. Med. 20, 682-688 (2014)). DeTiN (v0) was used to identify tumor in normal contamination. Oncotator was used to annotate identified variants (http://www.broadinstitute.org/cancer/cga/oncotator), and ABSOLUTE was used to estimate mutational clonality (Carter, S. L. et al. Nat Biotechnol 30, 413-421 (2012)). Depth of coverage was determined using the GATK DepthOfCoverage module. Mutational signature analysis was performed as previously described (Kim, J. et al. Nature genetics 48, 600-606 (2016)). Neoantigen prediction was inferred using Polysolver and binding affinity was predicted using NetMHCpan (v3.0).

All further statistical analyses were done in R (v.3.3.2)

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

No unique materials were used.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

No antibodies were used.
10. Eukaryotic cell lines
   a. State the source of each eukaryotic cell line used.
      No eukaryotic cell lines were used.
   b. Describe the method of cell line authentication used.
      No eukaryotic cell lines were used.
   c. Report whether the cell lines were tested for mycoplasma contamination.
      No eukaryotic cell lines were used.
   d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.
      No eukaryotic cell lines were used.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals
    Provide details on animals and/or animal-derived materials used in the study.
    No animals were used in this study.

Policy information about studies involving human research participants

12. Description of human research participants
    Describe the covariate-relevant population characteristics of the human research participants.
    Our final cohort consisted of 249 patients, for whom population characteristics were derived from published cohort information. Median age was 63 (range 18-86), and 95 were female (38%). Smoking status was available for 63 patients, of whom 15 were current smokers (24%), 29 (46%) were former smokers, and 19 (30%) were never smokers. These studies were conducted in accordance with the Declaration of Helsinki and approved by the Dana-Farber Cancer Institute Institutional Review Board (Protocols 11-104, 05-042, 02-180, 09-472, 02-021, 15-330).