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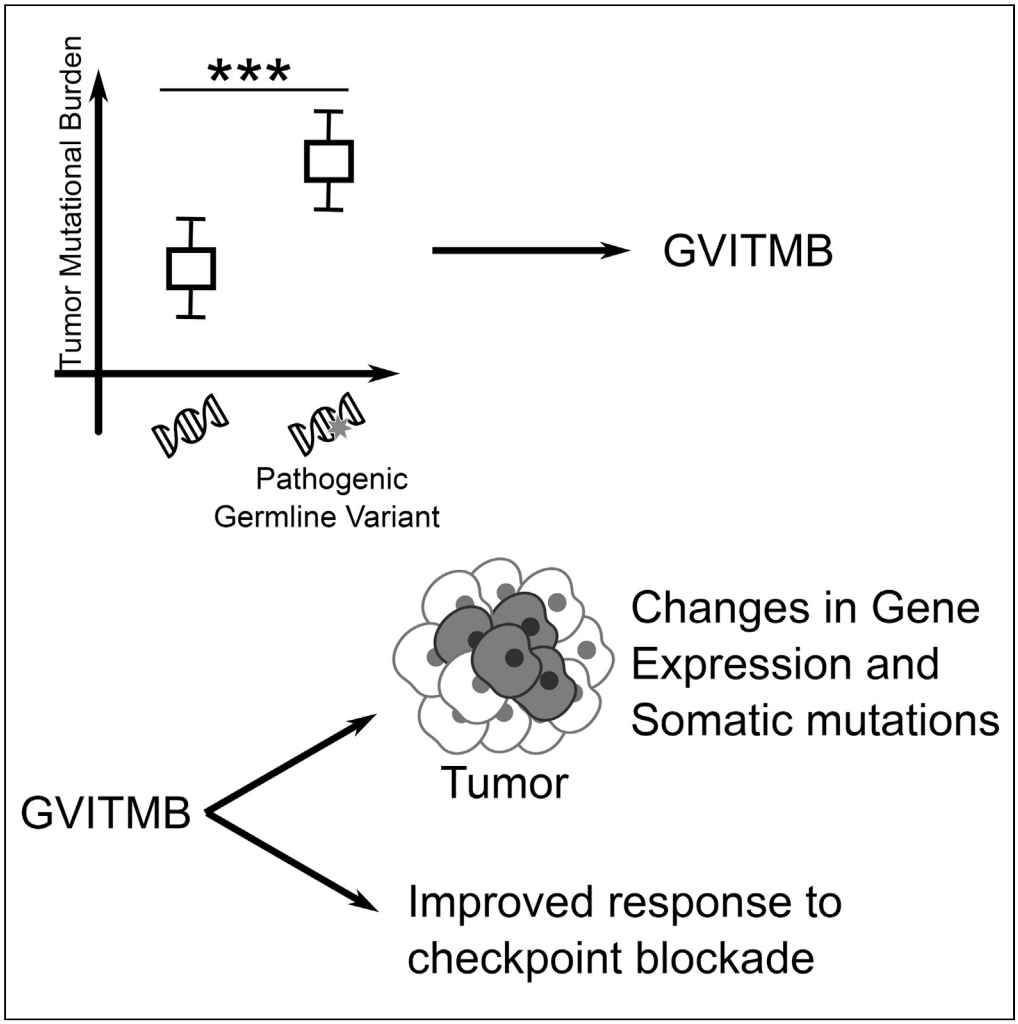

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HIGHLIGHTS

GVITMB were found in 7 genes and 38 gene sets

GVITMB influence the somatic mutation and gene expression profiles of tumors

GVITMB predict immune checkpoint inhibitory efficacy in SKCM

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Germline variants predictive of tumor mutational burden and immune checkpoint inhibitor efficacy

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SUMMARY

High tumor mutational burden (TMB) is associated with response to checkpoint blockade in several cancers. We identify pathogenic germline variants associated with increased TMB (GVITMB). GVITMB were found in 7 genes using a pan-cancer approach (*APC, FANCL, SLC25A13, ERCC3, MSH6, PMS2,* and *TP53*) and 38 gene sets (e.g., those involved in DNA repair and programmed cell death). GVITMB were also associated with mutational signatures related to the dysfunction of the gene carrying the variant, somatic mutations that further affect the gene or pathway with the variant, or transcriptomic changes concordant with the expected effect of the variant. In a validation cohort of 140 patients with cutaneous melanoma, we found that patients with GVITMB had prolonged progression-free survival (p = 0.0349, hazard ratio = 0.688) and responded favorably (p = 0.0341, odds = 1.842) when treated with immune checkpoint inhibitors. Our results suggest that germline variants can influence the molecular phenotypes of tumors and predict the response to immune checkpoint inhibitors.

INTRODUCTION

The explosion of massively parallel sequencing data has helped to identify rare germline variants that cause or contribute to disease (Sanderson et al., 2019; Vaske et al., 2019). In oncology, it is well-established that patients with germline variants in genes mutated in certain genetic syndromes, such as Lynch syndrome, Li-Fraumeni syndrome, von Hippel-Lindau syndrome, and Fanconi anemia, are at much higher risk of acquiring cancer (Ellrott et al., 2018; Kamps et al., 2017). Although individuals with these pathogenic germline variants are generally screened more aggressively, clinical management of patients with these germline variants is not always differentiated from the management of patients without these pathogenic variants (Ballinger et al., 2017; Lindor et al., 2006; Maher et al., 1990). This has begun to change. For example, patients with Lynch syndrome have pathogenic germline variants in mismatch repair genes, such as *MSH2, MSH6, PMS2,* and *MLH1,* and their tumors exhibit higher levels of microsatellite instability. As a consequence, patients with Lynch syndrome are more likely to respond to immune checkpoint inhibitors such as pembrolizumab (Snyder et al., 2014; Van Allen et al., 2015) (Le et al., 2017).

We have reported that germline variants affect tumor progression across a large spectrum of cancers through the analysis of common germline variants with a minor allele frequency greater than 5% in the general population (Chatrath et al., 2019, 2020). In this study, we analyze rare pathogenic germline variants to identify germline variants associated with increased tumor mutational burden (GVITMB) to test whether these germline variants increase the likelihood of a patient responding to immune checkpoint inhibitors (Keenan et al., 2019; Liu et al., 2019; Miao et al., 2018). After identifying the set of pathogenic germline variants predictive of tumor mutational burden (TMB), we demonstrate that they predict responsiveness to immune checkpoint inhibitors in a cohort of 140 patients with skin cutaneous melanoma.

RESULTS

Germline variants can be analyzed using pan-cancer or gene set-level approaches

Huang et al. had previously described 435 rare pathogenic germline variants that were found in the patients in The Cancer Genome Atlas (TCGA) (Huang et al., 2018). Briefly, all somatic variants were scored based on the American College of Medical Genetics and Genomics and the Association for Molecular Pathology

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(ACMG-AMP) guidelines developed for rare variants in cancer and variants known to be pathogenic in ClinVar and curated databases were labeled as pathogenic. The majority of these pathogenic germline variants were predicted to functionally perturb known tumor suppressor genes or oncogenes. Before identifying which pathogenic germline variants contribute to elevated TMB, we first evaluated whether we were able to identify GVITMB based in individual genes in individual cancers. We set a modest threshold requiring at least five patients in the cancer cohort to have a pathogenic germline variant in a given gene.

We utilized four approaches to identify germline variants associated with increased TMB. (1) We tested individual genes for association with TMB in individual cancers, testing a total of 13 unique genes (Figure 1A). (2) We pooled all the patients in TCGA together, and by doing so we were now able to test 73 total genes for the presence of GVITMB (Figure 1B). (3) We grouped the pathogenic germline variants by gene set to identify gene sets carrying GVITMB in individual cancers (Figure 1C). (4) Finally, we repeated the analysis in (3) but after grouping all cancers together (Figure 1D). Our overall methodology is summarized in Figure 2.

Calculation of tumor mutational burden

Overall TMB, nonsynonymous TMB, and clonal nonsynonymous TMB have previously been reported to be associated with favorable response in patients treated with immune checkpoint inhibitors (Keenan et al., 2019; Miao et al., 2018). We, therefore, calculated these three metrics of TMB for each patient in TCGA and normalized them to per megabase (MB) based on the total number of sites in each patient wherein we were sufficiently powered to call a somatic mutation. This normalization accounted for the coverage at each site in the exome and the purity and ploidy of each tumor. All metrics of TMB were highly correlated to each other (Spearman's rho >0.90 for all pairs, Figure 3A), and we present the normalized distribution of TMB by cancer in Figure 3B. We used clonal nonsynonymous TMB per MB as our dependent variable for this study as it has been shown to have a better association with immune checkpoint inhibitor responsiveness (Keenan et al., 2019; Miao et al., 2018).

Pan-cancer identification of individual genes associated with TMB

We identified seven genes that when perturbed by a pathogenic germline variant are associated with elevated TMB (Figure 4A, Table 1). Three of these genes (*APC*, *FANCL*, and *SLC25A13*) were determined to be significant after multiple hypothesis testing correction (adjusted p value <0.05). However, later in this study we also characterize the four genes (*ERCC3*, *MSH6*, *PMS2*, and *TP53*) that did not reach the significance threshold of an adjusted p value < 0.05 even though they crossed the raw p value threshold of <0.05 because they have well-known roles in DNA repair.

Identification of gene sets carrying GVITMB in individual cancers

We identified significant associations of pathogenic germline variants in gene sets and TMB in Colon Adenocarcinoma (COAD), Skin Cutaneous Melanoma (SKCM), and Uterine Corpus Endometrial Carcinoma (UCEC) (Figure 4B, Table 2, list of perturbed genes in Table S1). Although each of the identified gene sets consisted of different and unique gene sets, the genes that empirically contributed to these gene sets sometimes overlapped in this analysis. We have therefore grouped gene sets for which the contributing genes entirely overlapped in this particular analysis. In total, we identified 29 associations (2 in COAD, 11 in SKCM, and 16 in UCEC). The significantly associated gene sets were primarily related to DNA damage and repair and cell cycle control.

Pan-cancer identification of gene sets carrying GVITMB

Last, we identified pathogenic germline variants associated with TMB using a pan-cancer approach in which the pathogenic germline variants were grouped by gene set (Figure 4C, Table 3, list of perturbed genes in Table S2). In total, we identified 12 significant associations. Several of the gene sets were related to *Wnt* signaling, and the pathogenic germline variants in *APC* greatly contributed to these associations, as described in our analysis of individual genes. One association was driven entirely by *SLC25A13* and had also been described in our previous analysis of individual genes. The other associations were related to apoptosis, cell cycle control, and DNA damage repair.

GVITMB influence somatic events

We next sought to characterize the somatic events associated with GVITMB. Several studies have suggested that germline variants influence somatic events (Carter et al., 2017; Chatrath et al., 2019, 2020; Chirita-Emandi et al., 2020). We found that patients with GVITMB in mismatch repair genes exhibited

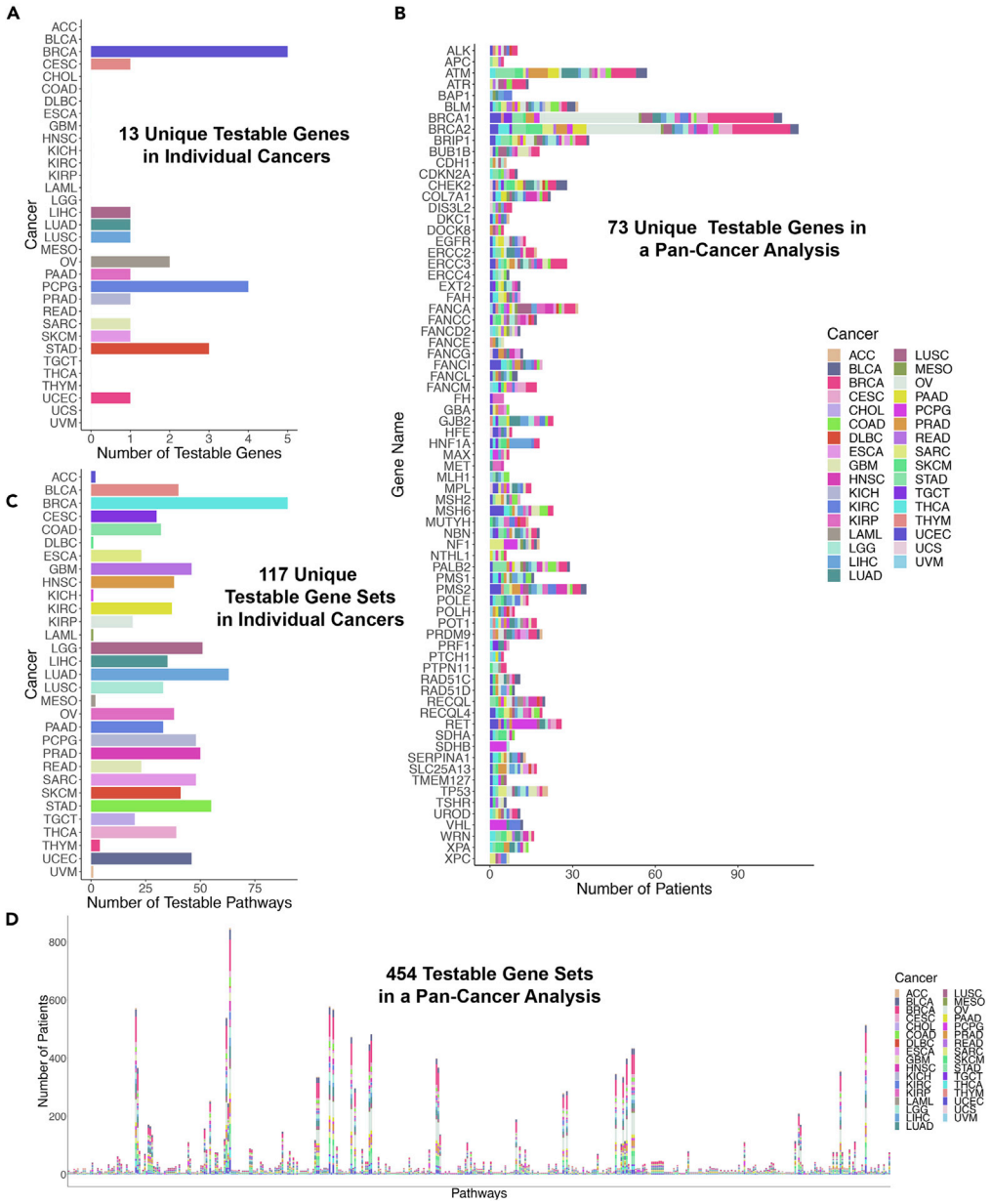

Figure 1. An overview of the number of genes or gene sets that could be tested with the threshold that the pathogenic germline variants must be present in five or more patients

(A) Number of testable genes in individual cancer types. This analysis was not performed due to the small number of testable genes.

(B) Number of patients with pathogenic variants in the indicated genes when patients with all cancers were pooled together. The stacked bars show the cancer types color coded as in the key. These patients were analyzed by Approach 1.

(C) Number of testable gene sets in each of the individual cancer types, analyzed by Approach 2.

(D) Number of patients carrying germline variants in the testable gene sets, analyzed by Approach 3. The stacked bars show the cancer types with pathogenic germline variants in a given gene set color coded as in the key.

enrichment of mutational signatures associated with mismatch repair gene dysfunction, suggesting exome-wide evidence of the dysfunction of these genes (Table 4).

We next tested whether the genes and gene sets perturbed by GVITMB were associated with somatic mutations in these same genes or gene sets. We controlled for TMB in all analyses to account for the general

Figure 2. A summary of the overall approach employed in this study

increase in somatic mutations in tumors with the GVITMB, along with controlling for tumor type and demographic factors. Patients with GVITMB in the mismatch repair gene *PMS2* were much more likely to exhibit somatic mutations in *PMS2* than patients without the GVITMB in *PMS2* (beta = 3.05, p value = 5.86E-5, adjusted p value=4.1E-4). We found that GVITMB in *ERCC3* or *TP53* were associated with an increased incidence of somatic mutations in gene sets that include *ERCC3* or *TP53*, respectively (Table S3). In addition, patients with SKCM with GVITMB in the disease gene set (a compilation of genes associated with human diseases) were more likely to acquire somatic mutations in other genes of the same gene set (beta=20.2, p value = 4.12E-6, adjusted p value=1.73E-4).

Finally, we tested for up- or downregulation of gene expression consistent with the expected effects of the GVITMB. We found that patients with GVITMB in genes regulating the G2-M checkpoint in UCEC exhibited upregulation of E2F target genes, suggesting upregulation of cell cycle activity (p value = 0.013).

GVITMB predict immune checkpoint inhibitory efficacy in SKCM

To test whether patients with SKCM with pathogenic germline variants in the gene sets that we had found to be associated with TMB in the TCGA dataset (Table 2) responded better to immune checkpoint inhibitors, we analyzed sequencing data from 140 patients with SKCM treated with either nivolumab or pembrolizumab (Liu et al., 2019). Given the relatively small sample size, we were not sufficiently powered to test individual gene sets for association with outcome. Of all the gene sets that contained GVITMB in SKCM

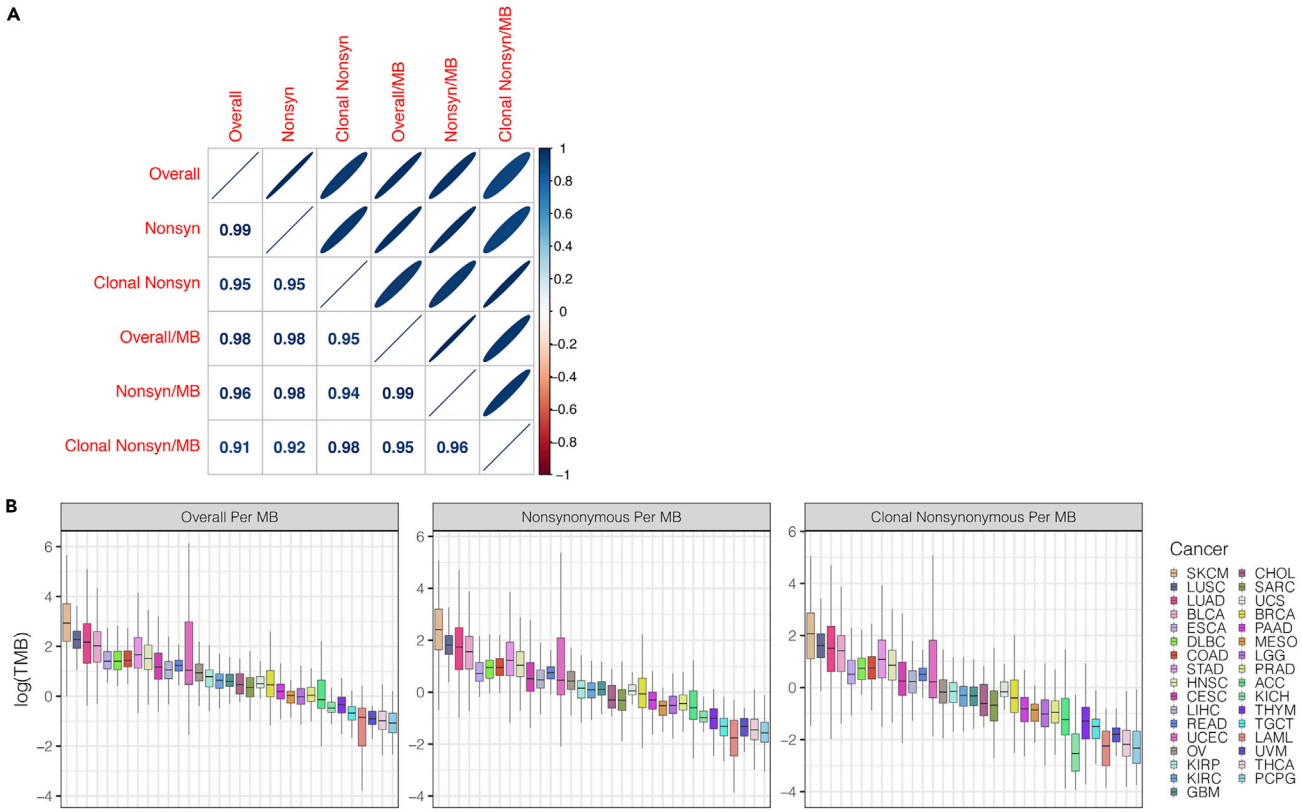

Figure 3. Calculated tumor mutational burden across cancers

(A) All six metrics of tumor mutational burden are highly correlated with each other.

(B) Overall TMB per megabase (MB), nonsynonymous TMB per MB, and clonal nonsynonymous TMB per MB across cancers.

(Table 2), only the disease gene set was sufficiently powered to detect an association with progression-free survival. Patients with pathogenic germline variants in the disease gene set exhibited prolonged progression-free survival ($p = 0.0245$, hazard ratio [HR] = 0.662) (Figures 5A and 5B) and were more likely to show a response to immune checkpoint inhibitors based on Response evaluation criteria in solid tumors (RECIST) criteria ($p = 0.0393$, odds = 1.781, ordering of categories was progressive disease, stable disease, partial response, and then complete response) (Figure 5C). Although patients with pathogenic germline variants had a higher median number of overall mutations, nonsynonymous mutations, and clonal nonsynonymous mutations, this difference was not statistically significant (Table S4, top three rows).

We were better powered to detect such an association by pooling all pathogenic germline variants found in the genes of the gene sets that we found to be associated with elevated TMB in SKCM the TCGA dataset (Figures 5D and Table 2). When tested, we found that patients with pathogenic germline variants in these genes exhibited favorable outcome and were less likely to progress (Figure 5E, $p = 0.0349$, HR = 0.688). Similarly, patients with pathogenic germline variants in these genes were more likely to exhibit a response to immune checkpoint inhibitors based on RECIST criteria (Figure 5F, $p = 0.0341$, odds = 1.842). Turning to TMB, we found that the median number of total mutations, nonsynonymous mutations, and clonal nonsynonymous mutations was greater in patients with pathogenic germline variants in genes in our gene set than patients without these pathogenic germline variants, although these differences were also not statistically significant (Table S4, lower three rows). Thus the GVITMB have a more significant effect on responsiveness than can be expected from the differences in TMB alone.

DISCUSSION

The widespread collection of sequencing data has enabled detailed study of rare genetic syndromes (Kamps et al., 2017; Sylvester et al., 2018). Although patients with pathogenic germline variants are often

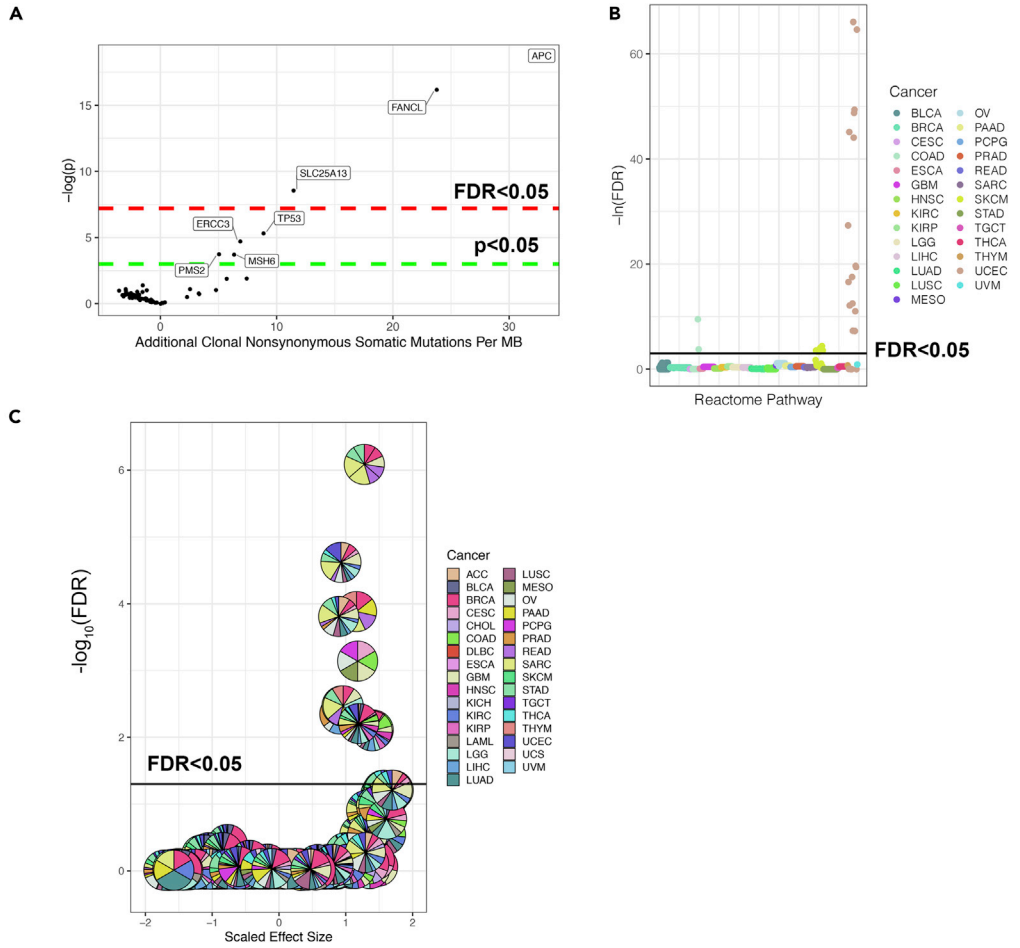

Figure 4. Manhattan plots summarizing the associations with clonal nonsynonymous tumor mutational burden per megabase

(A–C) We identified associations with elevated tumor mutational burden in (A) genes perturbed by pathogenic germline variants using a pan-cancer approach, (B) gene sets perturbed by pathogenic germline variants in individual cancers, and (C) gene sets perturbed by pathogenic germline variants using a pan-cancer approach. For each gene set, the fraction of patients with a particular cancer carrying a pathogenic germline variant is indicated by the color code.

screened more aggressively for cancer, clinical guidelines for these patients have only changed in a few circumstances (Le et al., 2017; Lindor et al., 2006). We had previously identified common germline variants associated with differences in patient outcome across a multitude of cancers, suggesting that germline variation contributes not only to cancer risk but also to tumor progression (Chatrath et al., 2019, 2020). In this study, we have identified pathogenic germline variants associated with TMB. Some of these associations were expected and confirmed existing hypotheses (e.g., mutations in known DNA repair genes such as *MSH6* and *PMS2*), whereas other associations (e.g., mutations in *SLC25A16*) are more surprising and can motivate future hypotheses. We identified molecular fingerprints of the effects of some of the pathogenic germline variants by analyzing RNA sequencing data and somatic mutation profiles. Our findings suggest that these pathogenic germline variants remain relevant after a patient has been diagnosed with cancer and may contribute to the molecular differences in tumors collected from patients with and without pathogenic germline variants.

After identifying the set of pathogenic germline variants associated with TMB in skin cutaneous melanoma, we showed that patients with these pathogenic germline variants exhibit prolonged progression-free survival and increased responsiveness to immune checkpoint inhibitors. Given the relatively small size of the validation cohort, our validation study had limited resolution because we were not adequately powered to test individual genes or gene sets. As the total amount of sequencing data from patients treated with

Table 1. A summary of the associations we found with elevated somatic mutation burden in individual genes using a pan-cancer approach

Gene	Number of patients with a PGV	Additional clonal nonsynonymous mutations per MB	p value	Adjusted p value
APC	5	32.51	7.910E-09	5.300E-07
FANCL	8	23.77	9.500E-08	3.180E-06
SLC25A13	17	11.46	1.938E-04	4.329E-03
TP53	16	8.87	4.897E-03	8.202E-02
ERCC3	23	6.87	9.044E-03	1.212E-01
MSH6	20	6.34	2.453E-02	2.348E-01
PMS2	32	5.05	2.367E-02	2.348E-01

PGV, Pathogenic Germline Variants.

immune checkpoint inhibitors continues to increase, our ability to identify individual genes and gene sets predictive of responsiveness will improve. In this study, we identify pathogenic germline variants associated with TMB as a proxy for immune checkpoint inhibitory efficacy, although determining the extent to which TMB is predictive of immune checkpoint inhibitor efficacy across all cancers is still an active area of research (Wood et al., 2020).

Tumors from patients with pathogenic germline variants in the mismatch repair genes *MSH6* and *PMS2* and in the mismatch repair pathway exhibit elevated TMB. We found enrichment in the contribution to these patients' somatic mutation profiles from COSMIC signatures related to mismatch repair. Germline mismatch repair deficiency has previously been associated with microsatellite instability and increased responsiveness to immune checkpoint inhibitors, and so these findings served as an important positive control in our study (Le et al., 2017).

Tumors with pathogenic germline variants in the nucleotide excision repair gene *ERCC3* were associated with elevated TMB in our study. Although a previous study showed that somatic mutations in the nucleotide base excision repair gene *ERCC2* likely contributes to increased TMB, no previous study has demonstrated an association between nucleotide excision repair gene perturbation and immune checkpoint inhibitor efficacy (Van Allen et al., 2014). We did not find a significant association between nucleotide excision repair pathway perturbation by pathogenic germline variants and TMB at the pathway level, suggesting that the contribution to TMB may be limited to select genes in the pathway.

We found patients with pathogenic germline variants in *APC*, which binds to beta-catenin and leads to its degradation, and genes involved with beta-catenin degradation to be associated with elevated somatic mutation burden. Aberrations to the *Wnt* signaling pathway are linked to the formation of many cancers (Anastas and Moon, 2013). Spranger et al. showed that non-T cell inflamed tumors exhibited high β-catenin signaling activity and reduced response to immune checkpoint blockade (Spranger et al., 2015). Further work is necessary to predict whether pathogenic germline variants in *APC* and genes involved with β-catenin degradation will be associated with increased or decreased response to immunotherapy, as the elevated TMB would be expected to increase efficacy, whereas the elevated β-catenin signaling would be expected to decrease efficacy.

Tumors from patients with pathogenic germline variants in *SLC25A13* exhibited elevated somatic mutation burden. This gene codes for a mitochondrial aspartate/glutamate transporter. Pathogenic germline variants in this gene are associated with the urea cycle disorder type II citrullinemia and neonatal intrahepatic cholestasis (Song et al., 2013). Lee et al. have previously shown that tumors exhibiting urea cycle dysfunction generate nitrogen metabolites, resulting in DNA damage and ultimately better response to immune checkpoint blockade (Lee et al., 2018). Lee et al.'s analysis focused on somatic urea cycle dysfunction, whereas our work suggests that germline urea cycle dysfunction may also be a marker for improved immune checkpoint blockade response.

FANCL is the E3 ubiquitin ligase subunit within the FA core complex that enhances the efficiency of FANCD2 monoubiquitination. FANCD2 participates in DNA damage recognition and repair. As the

Table 2. A summary of the associations we found with elevated somatic mutation burden in individual gene sets in individual cancers

Gene set	Cancer	Patients with PGV	Additional clonal nonsynonymous mutations per MB	p value	Adjusted p value
TP53 regulates transcription of DNA repair genes	UCEC	14	38.78	8.712E-31	2.004E-29
DNA repair	UCEC	31	25.71	7.358E-30	8.462E-29
Transcriptional regulation by TP53	UCEC	20	27.86	4.704E-23	3.607E-22
Generic transcription pathway	UCEC	22	26.34	1.132E-22	6.508E-22
Disease	UCEC	12	34.18	5.317E-21	2.446E-20
Gene expression transcription	UCEC	24	23.87	1.828E-20	7.009E-20
Mismatch repair, diseases of mismatch repair (MMR)	UCEC	7	34.55	3.925E-13	1.290E-12
Sumoylation	UCEC	6	31.43	9.928E-10	2.854E-09
Deubiquitination	UCEC	6	31.15	1.396E-09	3.568E-09
Fanconi anemia pathway	UCEC	8	25.54	1.040E-08	2.393E-08
DNA double-strand break response	UCEC	7	26.42	2.932E-08	6.130E-08
G2 M checkpoints, G2 M DNA damage checkpoint, regulation of TP53 activity, regulation of TP53 activity through phosphorylation	UCEC	9	20.00	1.958E-06	3.753E-06
Post-translational protein modification	UCEC	9	19.61	3.065E-06	5.423E-06
Cell cycle checkpoints	UCEC	10	17.62	9.955E-06	1.635E-05
Disease	COAD	8	20.53	4.064E-06	7.315E-05
DNA double-strand break repair	UCEC	15	11.48	4.291E-04	6.580E-04
Cell cycle	UCEC	15	11.37	4.835E-04	6.950E-04
DNA repair	SKCM	30	7.89	6.214E-04	1.243E-02
Disease	SKCM	6	16.17	1.680E-03	1.680E-02
Cell cycle	SKCM	20	8.43	2.805E-03	1.870E-02
Generic transcription pathway, gene expression transcription	COAD	15	9.86	2.467E-03	2.220E-02
Cell cycle checkpoints	SKCM	12	9.57	8.553E-03	2.851E-02
Regulation of TP53 activity	SKCM	11	10.38	6.340E-03	2.851E-02
DNA double-strand break repair	SKCM	19	7.72	7.643E-03	2.851E-02
Homology-directed repair (HDR) through homologous recombination (HRR)	SKCM	17	7.85	1.027E-02	2.903E-02

(*Continued on next page*)

Table 2. *Continued*

Gene set	Cancer	Patients with PGV	Additional clonal nonsynonymous mutations per MB	p value	Adjusted p value
Resolution of D-loop structures, resolution of D-loop structures through synthesis-dependent strand annealing (SDSA), homologous DNA pairing, and strand exchange	SKCM	16	7.96	1.161E-02	2.903E-02
Generic transcription pathway, gene expression transcription	SKCM	18	6.90	2.029E-02	3.568E-02
G2 M checkpoints, G2 M DNA damage checkpoint, regulation of TP53 activity through phosphorylation	SKCM	10	9.52	1.696E-02	3.568E-02
Transcriptional regulation by TP53	SKCM	16	7.44	1.827E-02	3.568E-02

pathogenic germline mutations in FANCL associated with TMB are predicted to be loss-of-function mutations, we hypothesize that they lower the efficacy of interstrand crosslink repair, affecting TMB.

High TMB has been associated with response to checkpoint blockade in several malignancies. However, the degree to which TMB changes over time, across anatomical sites, and with intervening treatment is still not clear. Studies have noted that tumor sampling from different anatomical sites may be associated with greater discrepancies in TMB calculations (Smithy et al., 2019). Efforts are ongoing to standardize TMB evaluation, which is needed to ensure reliability, reproducibility, and clinical utility (Galuppini et al., 2019). Compared with TMB, germline variants are relatively simpler to detect, annotate, score, and classify (Huang et al., 2018). Furthermore, they do not change during the course of the disease. It remains to be evaluated if they have additional value as a biomarker beyond that is provided by TMB, but our analyses suggest that they should be viewed as a biomarker candidate that can provide a robust and reproducible signal.

Overall, the results of our analysis suggest that understanding the germline contribution to somatic events could inform clinical therapy decisions (Carter et al., 2017; Menden et al., 2018). In this study, we have shown that pathogenic germline variants inform TMB and that these sets of pathogenic germline variants can be used to predict immune checkpoint inhibitor efficacy in patients with skin cutaneous melanoma. Future studies of germline variants in cancer will likely continue to illuminate areas in which clinical management can be further personalized based on an understanding of a patient's germline variants.

Limitations of the study

In this study, we used the TCGA data to identify pathogenic germline variants that are associated with increased tumor mutation burden (GVITMB). More than 80% of the patients in TCGA are of European ancestry, so it remains to be seen whether these associations will be replicated in a more diverse cohort. For the association analysis, we collapse the pathogenic variants in genes and gene set with the assumption that all pathogenic germline variants contribute toward increased TMB. It is likely that using adaptive burden association tests could increase our power to determine the associations, but that would come at the expense of interpretability. Using a second SKCM dataset, we were able to show that GVITMB have prognostic value, but it still needs to be determined whether GVITMB offer additional prognostic value beyond TMB. However, GVITMB do offer some advantages, as we highlight in the discussion, and should be considered as possible biomarker candidates in future studies.

Resource availability

Lead contact

Further information and questions should be directed to and will be fulfilled by the lead contact, Anindya Dutta (ad8q@virginia.edu).

Table 3. A summary of the associations we found with elevated somatic mutation burden in individual gene sets using a pan-cancer approach

Gene set	Number of patients with PGV	Additional clonal nonsynonymous mutations per MB	p value	Adjusted p value
Degradation of β-catenin by the destruction complex	5	32.51	7.907E-09	8.223E-07
β-catenin phosphorylation cascade, disassembly of the destruction complex and recruitment of axin to the membrane, signaling by WNT in cancer, phosphorylation site mutants of CTNNB1 are not targeted to the proteasome by the destruction complex	5	32.51	7.907E-09	8.223E-07
Ovarian tumor domain proteases	22	13.70	3.466E-07	2.403E-05
Deactivation of the β-catenin transactivating complex	7	22.42	2.517E-06	1.309E-04
Programmed cell death	28	11.03	3.729E-06	1.551E-04
Regulation of kit signalling	5	23.99	2.086E-05	7.231E-04
Apoptotic cleavage of cellular proteins, apoptotic execution phase	11	14.55	1.299E-04	3.378E-03
Signaling by WNT, TCF-dependent signaling in response to WNT	10	15.31	1.241E-04	3.378E-03
Mitochondrial protein import, gluconeogenesis, glucose metabolism, aspartate and asparagine metabolism, protein localization	17	11.46	1.938E-04	4.480E-03
Disease	211	3.17	2.993E-04	6.226E-03
Mismatch repair	63	5.63	4.116E-04	7.782E-03
Diseases of mismatch repair (MMR)	62	5.62	4.615E-04	7.999E-03

Materials availability

This study did not generate new unique reagents.

Data and code availability

All scripts used for analyses are available at https://github.com/achatrath/GermlineSomaticMutationBurden.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

Table 4. Mutational signature results concordant with the expected effects of the pathogenic germline variants

Gene or gene set	Cancer	Mutational signature	Fold enrichment	p value
MSH6	Pan-cancer	44	3.83	3.11E-03
Mismatch repair	UCEC	20	2.16	2.90E-02
Mismatch repair	Pan-cancer	20	2.16	2.13-03
Mismatch repair	Pan-cancer	26	1.58	3.48E-02
Mismatch repair	Pan-cancer	44	2.89	8.38E-06

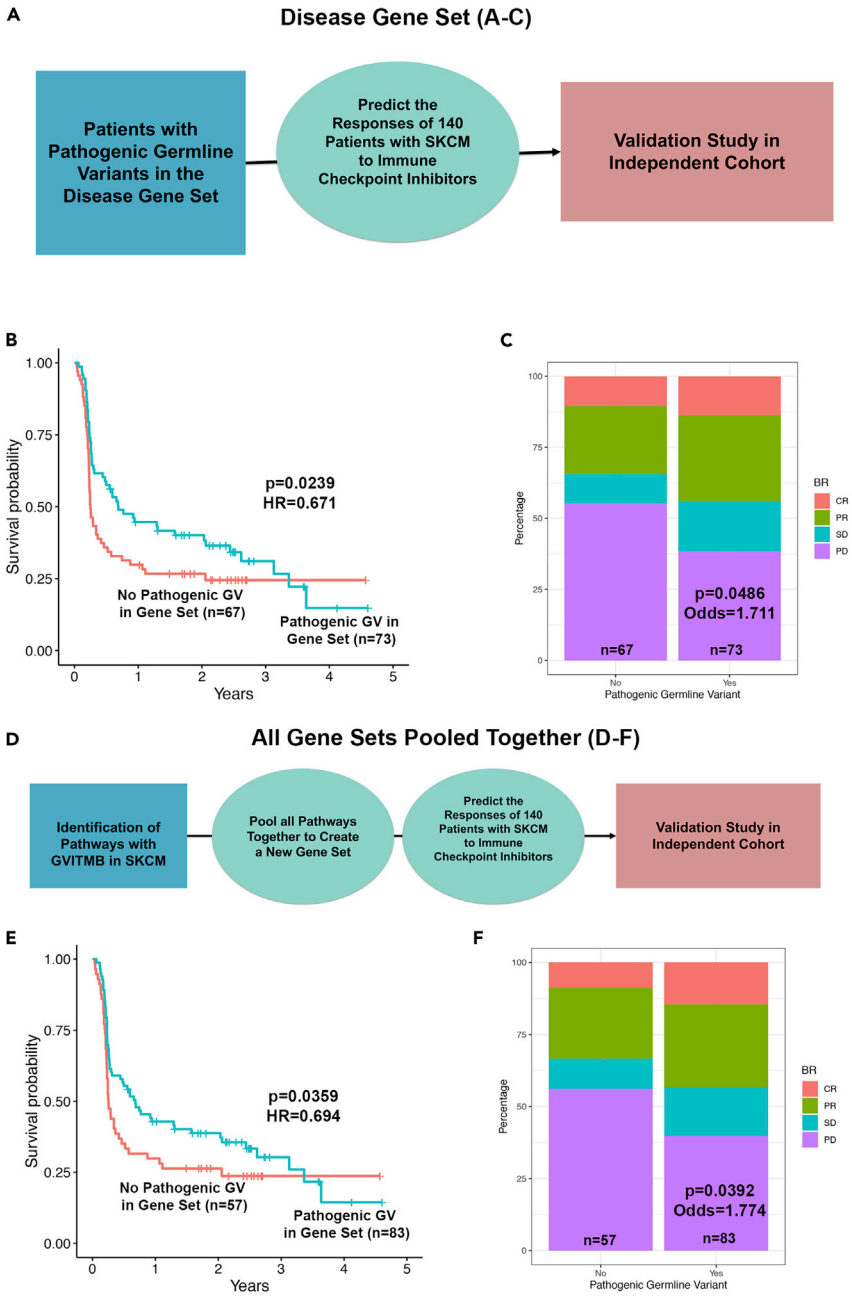

Figure 5. Pathogenic germline variants predict immune checkpoint inhibitor efficacy in an independent cohort of 140 patients with skin cutaneous melanoma treated with immune checkpoint inhibitors

(A–C) Patients with pathogenic germline variants in the (A) disease gene set exhibit (B) prolonged progression-free survival and (C) are more likely to respond to immune checkpoint inhibitors.

(D–F) We (D) pooled all gene sets with GVITMB in SKCM together and found that patients with germline variants in these gene sets exhibited (E) prolonged progression-free survival and are (F) more likely to respond to immune checkpoint inhibitors.

Abbreviations: PD, progressive disease; SD, stable disease; PR, partial response; CR, complete response.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.102248.

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AUTHOR CONTRIBUTIONS

Conceptualization, A.C. and A.D.; methodology, A.C., A.R., and A.D.; software, formal analysis, investigation, writing – original draft, visualization, and data curation, A.C.; resources and funding acquisition, A.D.; writing – review & editing, all authors; supervision and administration, A.D. and A.R.

DECLARATION OF INTERESTS

As required by our employer, the University of Virginia, we have declared the invention to the University, based on which the University has applied for a provisional patent application. All three authors of this manuscript will be the inventors declared in the patent application.

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Supplemental information

Germline variants predictive of tumor mutational burden and immune checkpoint inhibitor efficacy

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Transparent Methods

Patient Data Availability

We downloaded the set of rare, pathogenic germline variants found in the patients in The Cancer Genome Atlas (TCGA) previously published by Huang et al. and the set of somatic mutations in these patients generated by Ellrott et al. (Ellrott et al., 2018; Huang et al., 2018). Clinical data for the TCGA patients were accessed from the TCGA pan-cancer clinical data resource (Liu et al., 2018). We used the calculated race of each patient from The Cancer Genome Ancestry Atlas to effectively control for genetic ancestry throughout our analyses (Yuan et al., 2018).

Calculating Tumor Mutational Burden (TMB)

We counted the number of somatic mutations called by Ellrott et al. in each patient in The Cancer Genome Atlas (Ellrott et al., 2018). This count depends on the number of sites in the exome where we are adequately powered to call a somatic mutation. We first determined the sequencing depth at each position in the exome for each patient using SAMtools (Lau et al., 2017; Li et al., 2009). We then estimated the power to detect a somatic mutation at each position in the exome for each patient using the R package PureCN utilizing the purity and ploidy previously reported by The Cancer Genome Atlas Pan-Cancer Atlas initiative (https://gdc.cancer.gov/about-data/publications/pancanatlas) (Riester et al., 2016). The somatic mutations had previously been categorized as synonymous or nonsynonymous by Ellrott et. al. (Ellrott et al., 2018). We classified the somatic mutations as clonal or subclonal by calculating the probability of observing the number of reads supporting the somatic mutation in the tumor based on a binomial distribution, given the total number of reads covering that

position. We assumed that the probability of a read supporting a clonal somatic mutation was equal to $\frac{1}{Ploidy*Tumor\ Purity}$. If the probability was less than 5% ($p<0.05$), we classified the somatic mutation as subclonal (Carter et al., 2012).

We calculated six metrics of tumor mutational burden. The first three were overall tumor mutational burden, nonsynonymous tumor mutational burden, and clonal nonsynonymous tumor mutational burden. These three metrics were normalized by the number of sites for which there was 80% or greater power to detect a somatic mutation. This enabled us to calculate overall tumor mutational burden per megabase, nonsynonymous tumor mutational burden per megabase, and clonal nonsynonymous tumor mutational burden per megabase. We used clonal nonsynonymous tumor mutational burden per megabase as our dependent variable for this study as clonal nonsynonymous TMB has been shown to be more closely associated with response to immune checkpoint inhibitors (Keenan et al., 2019; Liu et al., 2019; Miao et al., 2018).

Identification of Genes with GVITMB

Across all of the TCGA patients, 132 unique genes contained at least one pathogenic germline variant. We limited our analysis only to genes with pathogenic germline variants in at least five different patients. Our results do not change substantially when we lower this threshold. However, setting the minimum threshold at five patients eliminates associations driven by a small number of patients, which could make them less compelling and more difficult to validate. We tested individual genes for association with clonal nonsynonymous tumor mutational burden per megabase, controlling for age, gender (if applicable), and calculated patient race. We tested a total of 13 unique genes.

We also looked for associations between individual genes and tumor mutational burden using a pan-cancer approach. We pooled all of the TCGA patients together and tested whether individual genes perturbed by pathogenic germline variants (presence or absence of a pathogenic germline variant) were associated with clonal nonsynonymous TMB per megabase using linear regression, controlling for tumor type, age, gender, and calculated patient race. We tested a total of 73 unique genes in this analysis. P-values were adjusted using the Benjamini-Hochberg procedure throughout this study.

Identification of Gene Sets with GVITMB

To study the association between pathogenic germline variants and tumor mutational burden in individual cancers, we grouped genes by gene sets. Gene sets perturbed by pathogenic germline variants in five or more patients were tested. Gene set annotation was downloaded from Reactome (Fabregat et al., 2018). We tested whether having a pathogenic germline variant in the gene set (presence or absence) was associated with clonal nonsynonymous TMB per megabase using linear regression, controlling for age, gender, and calculated patient race. We tested a total of 117 unique gene sets. Finally, we performed a pan-cancer analysis of gene sets associated with clonal nonsynonymous TMB per megabase using the same approach, controlling for tumor type, age, gender, and calculated patient race. We tested a total of 454 unique gene sets in this analysis. While each gene set included in these analyses is unique, some of the gene sets have overlapping sets of genes (**Table 2-3** and **Table S1-2**).

Gene Set Enrichment Analysis

We performed gene set enrichment analysis to test for upregulation or downregulation of RNAs in specific gene sets in patients with GVITMB. To do this, we downloaded the previously released RNA-sequencing quantification files for each patient generated by the TCGA research network (https://portal.gdc.cancer.gov/). We then excluded genes with a median expression level of <1 FPKM across the patient cohort being tested. The expression values of the remaining genes were then standardized to have a mean of 0 and a standard deviation of 1. We ranked the genes by coefficients after measuring the association between the expression of each gene and the status of the GVITMB under study using logistic regression, controlling for tumor type, age, gender, and calculated patient race. We used these ranked gene lists to perform Gene Set Enrichment Analysis (Subramanian et al., 2005).

Mutational Signature Analysis

We hypothesized that the tumors of some of the patients with GVITMB would exhibit enrichment of mutational signatures. We downloaded all single base substitution signatures from COSMIC (Tate et al., 2019). We determined the optimal contribution of COSMIC signatures to reconstruct the mutational profile observed in each of the patients in TCGA using the R package “MutationalPatterns” (Blokzijl et al., 2018). We converted the contribution values to percentages, such that the sum of the percent contributions of all the COSMIC signatures for each patient was equal to 100%.

We evaluated whether a COSMIC signature is enriched in tumors with a GVITMB by testing for the association between the percent contribution of a signature and the presence or absence of the GVITMB, controlling for tumor type, age, gender, and calculated patient race.

Increased Susceptibility to Mutations in Driver Genes and in the Same Gene Set

To ask if the GVITMB influenced the somatic mutations acquired by the patient, we calculated the number of "probably damaging" somatic mutations in each gene in each patient, as classified by Ellrott et al. (Ellrott et al., 2018). We tested whether the chance of observing a "probably damaging" somatic mutation in the same gene as the GVITMB was more likely in patients with the GVITMB using logistic regression, controlling for tumor type, age, gender, calculated patient race, and clonal nonsynonymous TMB per megabase. We also tested whether the mutational burden in genes of the gene set that the GVITMB was found in differed based on the germline variant status using linear regression, controlling for tumor type, age, gender, calculated patient race, clonal nonsynonymous TMB per megabase and the number of sites for which we were sufficiently powered to call somatic mutations. We controlled for the TMB in both these analyses to test if the mutation burden in these somatically mutated genes was higher than that could be explained by the increase in the overall TMB of the patient.

Validation in an Independent Cohort of Patients with Skin Cutaneous Melanoma Treated with Immune Checkpoint Inhibitors

As part of our analysis, we had identified patients with pathogenic germline variants predictive of TMB in patients with Skin Cutaneous Melanoma (SKCM). We hypothesized that patients with this set of pathogenic germline variants would exhibit a favorable response to immune checkpoint inhibitors (Keenan et al., 2019; Liu et al., 2019; Miao et al., 2018; Van Allen et al., 2015; Van Allen et al., 2014). We, therefore, analyzed sequencing data from 140 patients with skin cutaneous melanoma treated

with immune checkpoint inhibitors (Liu et al., 2019). Although there are a total of 144 patients in this cohort, we only included 140 patients in this study as 4 patients had a "mixed response" to treatment that was not clearly categorized.

We downloaded the raw reads from the non-tumor samples from dbGAP (accession number: phs000452.v3.p1) using the SRA toolkit (http://ncbi.github.io/sra-tools/). The data was aligned and variant called according to GATK best practices (Liu et al., 2019; Van der Auwera et al., 2013). Germline variants were categorized as pathogenic using CharGer (Scott et al., 2019).

We tested whether we were sufficiently powered to detect differences in progression free survival based on the status of the pathogenic germline variants we had identified assuming a hazard ratio of 2 using the "powersurvepi"(https://cran.r-project.org/web/packages/powerSurvEpi/powerSurvEpi.pdf) R package. We were not sufficiently powered to detect associations at the level of individual genes or gene sets. We, therefore, combined all of the gene sets where GVITMB were found in SKCM to create a test gene set for responsiveness to immune checkpoint inhibitors. We were adequately powered to perform the analysis in this larger cohort, as the probability of detecting an association assuming a hazard ratio of 2 was 92.5%. We tested whether GVITMB in this test gene set were associated with progression free survival using Cox regression, controlling for age, gender, treatment type (Nivolumab or Pembrolizumab), prior treatments, and whether or not the patient had brain lymph node, lung, liver, or bone metastases. Cox regression was performed using the "survival" and "survminer" R packages. We tested whether the patients with pathogenic germline variants in the test gene set were associated with increased responsiveness to immune checkpoint

inhibitors based on RECIST criteria using ordinal logistic regression in R. The responses were ordered as follows: progressive disease, stable disease, partial response, and complete response. Our null hypothesis was that the patients with pathogenic variants in genes included in this gene set would not exhibit a favorable response to immune checkpoint inhibitors. To test that hypothesis, we performed one-sided statistical tests when testing for an association between progression free survival and response based on RECIST criteria and germline variants in our test gene set.

Software

Computation was performed using R version 3.5.2. The R packages “ggplot2” and “scatterpie” were used to generate the figures in this manuscript.