

ORIGINAL RESEARCH

# Association of differential expression of immunoregulatory molecules and presence of targetable mutations may inform rational design of clinical trials

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Available online xxx

**Background:** Immune checkpoint inhibitors (ICIs) and genomic biomarker-driven targeted therapies have revolutionized the modern oncologic treatment arsenal. The next step has been to combine targeted agents and ICIs. In doing so, some combination regimens may be more logical than others.

**Patients and methods:** Whole-exome and whole-transcriptome sequencing were performed on 2739 unselected later-stage clinical cases from 24 solid tumor subtypes in the NantHealth database, and data were also curated from 5746 similarly sequenced patients across 28 solid tumor subtypes in The Cancer Genome Atlas (TCGA). Significant differential expression of 10 immunoregulatory molecules [IRMs (genes)] was analyzed for association with mutant versus wild-type genes.

**Results:** Twenty-three significant associations between currently actionable variants and RNA-expressed checkpoint genes were identified in the TCGA cases; 10 were validated in the external cohort of 2739 clinical cases from NantHealth (*P* values were adjusted using Benjamini–Hochberg multiple hypothesis correction to reduce false-discovery rate). Within the same 5746 TCGA profiles, 2740 TCGA patients were identified as having one or more potentially oncogenic single-nucleotide variant (SNV) mutation within an established 50-gene hotspot panel. Of the 50 genes, SNVs within 15 were found to be significantly associated with differential expression of at least one IRM after adjusting for tissue enrichment; six were confirmed significant associations in an independent set of 2739 clinical cases from NantHealth.

**Conclusions:** Logically combining ICIs with targeted therapies may offer unique treatment strategies for patients with cancer. The presence of specific mutations impacts the expression of IRMs, an observation of potential importance for selecting combinations of gene- and immune-targeted therapeutics.

**Key words:** next-generation sequencing, genomic medicine, precision oncology, immunoregulatory molecules, checkpoint molecules, oncogenes, targetable mutations

## INTRODUCTION

Immune checkpoint inhibitors (ICIs) are an important new part of the armamentarium against cancer. However, >80% of unselected patients do not respond.<sup>1</sup> Currently, many clinical trials focus on the use of ICIs in combination with standard chemo- (or other) therapies, but many of these

trials fail, at least in part due to lack of optimal patient selection.<sup>2</sup> Here, we sought to identify which checkpoint genes are differentially expressed in the presence of therapeutically targetable DNA mutations in order to facilitate the rational design of clinical trials for ICI combination therapies.

The current standard of care in cancer therapy is founded on nonpersonalized targeting of tissue type. Several meta-analyses totaling ~85 000 patients have shown that personalized (biomarker-based) therapies improve outcomes (especially if the biomarker is a genomic one) as compared with nonbiomarker-selected therapies.<sup>3–5</sup> Such findings have encouraged widespread interest in a precision approach to cancer therapy based on determining the presence of actionable cancer driver genes and/or expression of checkpoint proteins.<sup>6</sup> Biomarkers for ICIs are also emerging as critically important.<sup>7–11</sup> As part of the rise of

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precision medicine,<sup>12-18</sup> there has been increased focus on immunoregulatory molecules (IRMs) such as programmed death receptor 1 (PD-1) or its ligand (PD-L1), CTLA4, TIM3, and others as targets for therapy.<sup>19</sup> Although drugs are currently in development for many of the IRMs as targets, targeting tumors that overexpress the various IRMs in conjunction with the associated gene alteration may theoretically provide clinical benefit for patients.

Efforts to identify molecular mechanisms underlying upregulation of IRMs in the presence of specific molecular alterations have shown, for instance, that inhibition of poly ADP-ribose polymerase (PARP) can lead to upregulation of PD-L1 via inactivation of GSK3 $\beta$ .<sup>20</sup> In addition, in colorectal cancers, upregulated FGFR2 is positively correlated with PD-L1 expression via the JAK/STAT pathway.<sup>21</sup> Another example lies with cyclin D and CDK4 kinase, which leads to instability of PD-L1 through CUL3-SPOP E3 ligase and is inherently related to APC degradation.<sup>22</sup> These examples help to elucidate the intricate relationship that somatic gene alterations play on the expression patterns of IRMs, most studied for PD-L1, but likely generalizable to other such IRMs.

The combination of targeted drugs and immunotherapies has had some of the most success in patients with renal cell carcinoma and hepatocellular carcinoma (HCC) to date.<sup>23-25</sup> Specifically, in patients with metastatic renal cell carcinoma, the combination of anti-PD-1 (pembrolizumab) with targeted therapies to vascular endothelial growth factor (VEGF; axitinib, lenvatinib, and cabozantinib) has shown superiority in patient outcomes compared with targeted therapy (sunitinib) alone.<sup>26-28</sup> Similarly, in patients with HCC, the combination of anti-PD-L1 (atezolizumab) and targeted therapy to VEGF (bevacizumab) had higher survival compared with targeted therapy (sorafenib) alone.<sup>29</sup> In HCC, the combination of targeted and immunotherapy continues to be adopted; in a recent phase III trial comparing the combination anti-PD-L1 plus targeted therapy to VEGF receptor (atezolizumab + cabozantinib) versus targeted therapy alone (sorafenib) showed longer survival for these patients (<https://www.cancernetwork.com/view/cabozantinib-plus-atezolizumab-extends-pfs-for-frontline-hcc-in-cosmic-312-trial>).

The aim of this study is to identify the relationship between somatic gene alterations and the expression of various IRMs. We hypothesized that expression of cancer driver genes may in turn affect levels of IRMs. If so, knowledge about any such associations could be exploited in patient selection for—or design of—clinical trials, including future trials of combined immuno- and genomically targeted therapies, as such candidates move forward.

## METHODS

Whole-exome sequencing variant calls and whole-transcriptome expression RNA sequencing (RNA-seq) were performed for 5746 patients across 28 solid tumor subtypes in The Cancer Genome Atlas (TCGA; [Supplementary](#)

[Table S1](#), available at <https://doi.org/10.1016/j.esmoop.2022.100396>). To identify therapeutically targetable variants associated with differential IRM expression, a curated database of sensitizing biomarkers was obtained from NantOmics. Examples of biomarkers and associated evidence are provided in [Supplementary Table S2](#), available at <https://doi.org/10.1016/j.esmoop.2022.100396>; gene coverage of curated citable findings is indicated in [Supplementary Figure S1](#), available at <https://doi.org/10.1016/j.esmoop.2022.100396>. For instance, information for BRAF alteration drug sensitivity would be curated.<sup>30-36</sup>

Significant differential expression of 10 IRM genes (CTLA4, FOXP3, LAG3, IDO, OX40, PD-1, PD-L1, PD-L2, TIGIT, TIM3) was analyzed in samples with mutant versus wild-type of different genes using Student's *t*-tests and corrected for multiple hypothesis testing using Benjamini–Hochberg adjustment. Associations between presence of targetable mutations and differential IRM expression that remained significant after correction were then validated in an external cohort of 2739 unselected later-stage clinical cases from 24 solid tumor subtypes in the NantHealth database with similarly profiled paired whole-exome sequencing and RNA-seq<sup>37</sup> (for methods, see<sup>37</sup>). These cases come from patients with different cancer types, including breast ( $n = 483$ ), colon ( $n = 239$ ), lung ( $n = 222$ ), pancreatic ( $n = 177$ ), and ovarian ( $n = 162$ ) cancers ([Supplementary Table S3](#), available at <https://doi.org/10.1016/j.esmoop.2022.100396>).

To expand the presented analysis beyond curated targetable variants to more exploratory targets, any variants within putative cancer driver genes were considered for their effect on IRM expression. Variants within the AmpliSeq 50-gene HotSpot v2 panel (<https://assets.thermofisher.com/TFS-Assets/LSG/brochures/Ion-AmpliSeq-Cancer-Hotspot-Panel-Flyer.pdf>) were pooled at the gene level and analyzed for correlation with IRM expression. As pooling variants at the gene level introduces more likelihood for confounding tissue-specific effects, tissue-specific effects on IRM expression were analyzed in addition to variant effects to ensure presence of driver-gene variants was tissue independent. For each gene, tissue-specific variant rate was assessed by Fisher's exact test and the most enriched tissue type was identified. The observed associations were then validated using the NantHealth external database of 2739 unselected clinical cancer cases as above.

## RESULTS

Our findings suggest that certain sensitizing mutations associate with upregulation or downregulation of specific IRMs. Whole-exome sequencing and RNA-seq were available for 5746 patients with 28 diverse types of solid malignancies from TCGA. These included (but are not limited to) breast invasive carcinoma ( $n = 976$ ), thyroid carcinoma ( $n = 401$ ), and prostate adenocarcinoma ( $n = 331$ ) cancers; as well as melanoma ( $n = 342$ ) and glioblastoma multiforme ( $n = 289$ ; [Supplementary Table S1](#), available at <https://doi.org/10.1016/j.esmoop.2022.100396>).

### Actionable variants associated with specific IRMs

Twenty-three significant associations between currently actionable variants and RNA-expressed checkpoint genes were identified in the TCGA cases (Figure 1A); 10 were validated in the external cohort of 2739 clinical cases from NantHealth, including the vemurafenib target BRAF V600E and increased PD-1, PD-L1, and CTLA4 expression (adjusted  $P = 2.4 \times 10^{-3}$ ,  $1.1 \times 10^{-23}$ ,  $6.6 \times 10^{-7}$ , respectively) but decreased IDO1 expression (adjusted  $P = 3.4 \times 10^{-6}$ ). *TIM3* was found significantly elevated in patients with lapatinib-sensitive EGFR G598V (adjusted  $P = 1.0 \times 10^{-5}$ ), most frequently in glioblastoma multiforme; and conversely suppressed in patients with FGFR3 S249C (adjusted  $P = 0.04$ ; Figure 1A and Supplementary Table S2, available at <https://doi.org/10.1016/j.esmooop.2022.100396>). Patients with PIK3CA E545K, mostly with cervical cancers, showed higher IDO1 expression (adjusted  $P = 3.3 \times 10^{-4}$ ), suggesting sensitivity to a combined regimen such as alpelisib (PIK3CA inhibitor) with epacadostat (IDO inhibitor). Further, as seen in Figure 1A, specific molecular alterations that are defined drivers and sensitive to targeted therapeutic inhibition may impact sample stratification, and elucidated 10 independently confirmed significant associations from two diverse datasets.

### Hotspot mutations associated with upregulation or downregulation of specific IRMs

To expand analysis beyond the limited set of previously curated mutations, presence of any potentially pathogenic mutations within cancer drive genes was considered. Within the same 5746 TCGA profiles, 2740 patients with TCGA solid tumor were identified to have at least one potentially oncogenic single-nucleotide variant (SNV) mutation within an established hotspot gene-panel—AmpliSeq 50-gene HotSpot v2 panel. Of the 50 studied driver genes, SNVs within 15 were found to be significantly correlated with differential expression of at least one IRM, including elevated CTLA4 and PD-1 with mutant *CDKN2A* (adjusted  $P = 1.93 \times 10^{-9}$ ), elevated IDO1 with *FBXW7* (adjusted  $P = 0.007$ ), and decreased PD-L1 with mutant *APC* (adjusted  $P = 0.02$ ; Figure 1B). Frequently, the effect size of having an SNV present was greater than specific tissue type; for example, expression of CTLA4 was more associated with *CDKN2A* mutants across tissue types than with head and neck squamous cell carcinomas ( $P = 1.93 \times 10^{-9}$  versus  $P = 4.17 \times 10^{-6}$ ), despite head and neck squamous cell carcinoma being identified as the tissue type most enriched for *CDKN2A* mutants (odds ratio 4.9,  $P = 4.3 \times 10^{-9}$ ; Supplementary Table S2, available at <https://doi.org/10.1016/j.esmooop.2022.100396>). Similarly, low PD-L1 was more associated with *APC* mutants across tissue types than within the diagnosis of colorectal carcinoma ( $P = 2.23 \times 10^{-2}$  versus  $P = 0.0559$ ).

Overall, 15 significant hotspot-gene mutations/IRM expression associations were identified; of these, 6 were validated in the NantHealth cohort (Figure 1B and Supplementary Table S4, available at <https://doi.org/10.1016/j.esmooop.2022.100396>).

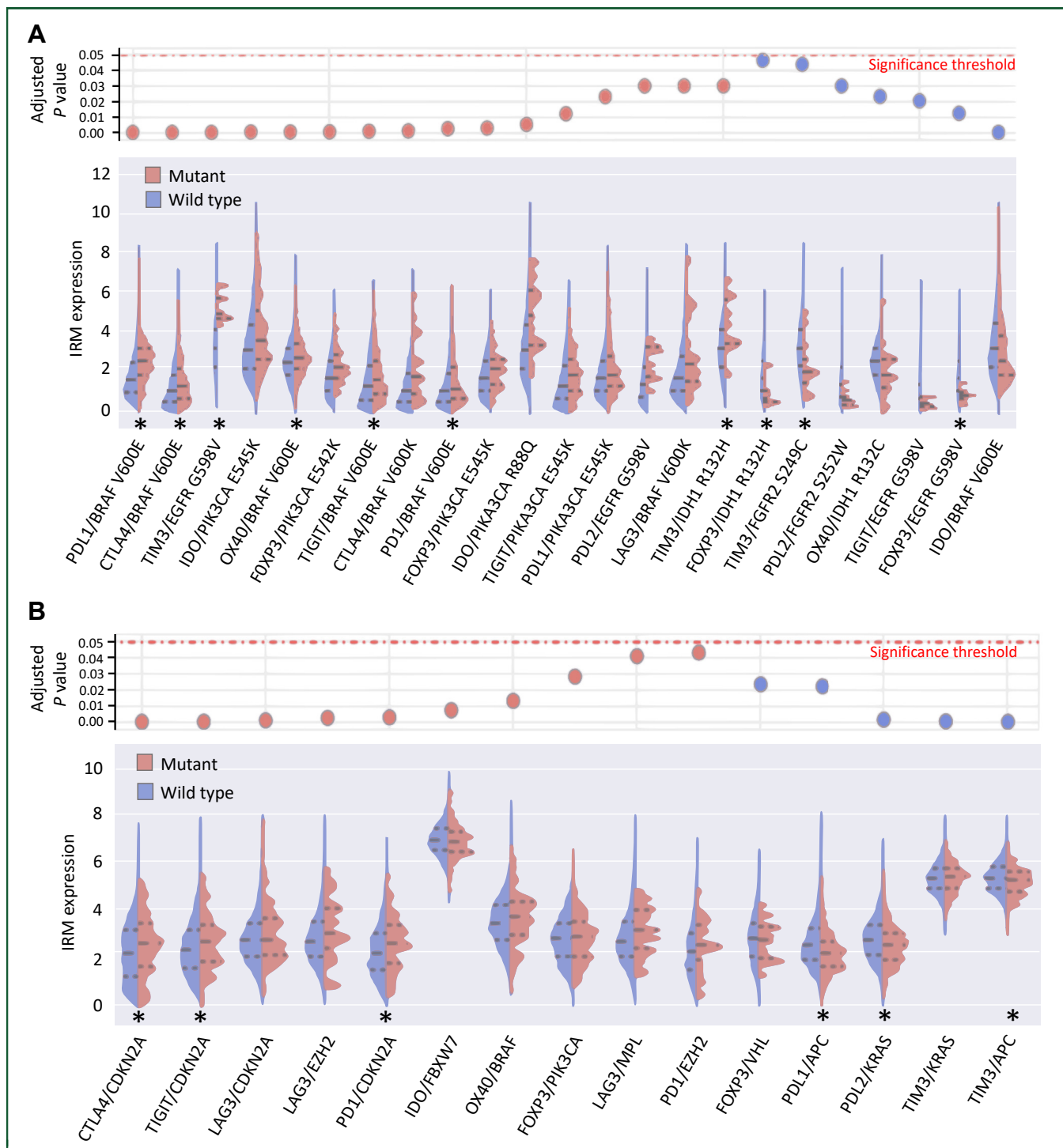
Specifically, it was validated that *CDKN2A* is associated with increased PD-1 and CTLA4, and that *KRAS* and *APC* are associated with decreased PD-L1/2 expression.

### DISCUSSION

Next-generation sequencing (NGS) data inform decisions for use of gene mutation-targeted therapies; use of similar analysis when paired with RNA-seq may support efforts to replace chemotherapy with more efficacious/safer combined immuno- and mutation-targeted therapies. Our findings here suggest future studies may result in the optimization of ICI-gene-targeted therapy trials.

Taken together, these data suggest that pembrolizumab or nivolumab may be a good combination with BRAF V600E inhibitors such as vemurafenib<sup>20-26</sup> or dabrafenib,<sup>27</sup> based on the significant associations found between BRAF V600E mutations and PD-L1 expression (Figure 1A).<sup>28,29</sup> The strategy of combining dabrafenib, trametinib, and pembrolizumab was shown to have a longer progression-free survival, duration of response, and overall survival compared with dabrafenib, trametinib, and placebo in patients with previously untreated BRAF V600E/K-mutated metastatic melanoma.<sup>38</sup> It is unclear if this same triplet therapy would yield benefit in other tumor histologies, but the association between BRAF V600E and PD-L1 expression is significant. Alternatively, adding a CTLA4 inhibitor such as ipilimumab to a triplet therapy may confer even more response in patients with BRAF V600E-mutated cancers, as CTLA4 is also upregulated in tumors bearing that genomic alteration (Figure 1A). Conversely, epacadostat (an IDO1 inhibitor) should possibly be avoided in BRAF V600E-mutated cancers because it is associated with downregulation of IDO (Figure 1A). An anti-TIM3 antibody such as TSR-022 may be effective in combination with lapatinib where EGFR G598V<sup>39</sup> mutations are sensitizing (Figure 1A). However, anti-TIM3 therapy may lack benefit with FGFR inhibitors, such as AZD4547 known to be effective in patients with FGFR3 S249C mutation, where TIM3 is downregulated in the presence of this mutation.<sup>40</sup> PIK3CA E545K mutations may be sensitive to epacadostat in addition to alpelisib due to the high expression of IDO found in tumors with this genomic alteration.<sup>41,42</sup> These premises however need to be prospectively tested. Ayers and colleagues<sup>43</sup> demonstrated that inflammation-associated T-cell gene expression signatures containing genes related to antigen presentation and cytokine expression were necessary to yield clinical benefit, which is relevant to our study in showing that somatic gene alterations can influence immune-modulating molecules.

Regardless of current therapeutic targetability, *APC* mutations appear associated with lower IRM expression, supporting a role for Wnt/ $\beta$ -catenin activation as an alternative to checkpoint expression in immune surveillance escape.<sup>44,45</sup> Conversely, *CDKN2A*/p16INK4a mutations are associated with positive expression of multiple IRM and may broadly serve as a sensitivity marker for ICI strategies.



**Figure 1.** (A) Immune checkpoint expression changes in the presence of sensitizing mutations. Twenty-three significant associations were found between defined targetable mutations and differential checkpoint expression from the 5746 patients in The Cancer Genome Atlas (TCGA) database. In the top panel, red dots indicate statistically significant upregulation of the immune regulatory molecule (IRM), while blue dots indicate significant downregulation in TCGA. X-axis represents the association between specific gene mutation and IRM. Y-axis is the level of expression of the checkpoint based on the specific gene mutation. For example, BRAF V600E mutations are significantly associated with having upregulation of PDL1 in RNA (far left-hand side) [as denoted with the red dots signifying upregulation of the IRM in RNA (see the upper part of the panel for dots)]. Further, BRAF V600E mutations are significantly associated with downregulation of IDO1 in RNA [far right-hand side, as denoted with the blue dots signifying downregulation of the IRM in RNA (see the upper part of the panel for dots)]. The asterisks ( $n = 10$ ) below represent confirmed significant associations in an independent set of 2739 clinical cases from NantHealth. P values were adjusted using Benjamini–Hochberg multiple hypothesis correction to reduce false-discovery rate. (B) Immune checkpoint expression changes in the presence of hotspot mutations. Fifteen associations were found between potentially pathogenic mutations and differential checkpoint expression after adjusting for tissue enrichment of the various genes. In the top panel, red dots indicate statistically significant upregulation of the IRM, while blue dots indicate significant downregulation in TCGA. Within 5746 TCGA profiles, 2740 TCGA solid-tumor samples had one or more mutation [single-nucleotide variant (SNV)] within an established hotspot gene-panel—AmpliSeq 50-gene HotSpot v2 panel. Of the 50 studied driver genes, SNVs within 15 were found to be significantly associated with differential expression of at least one IRM. For example, CDKN2A mutations, regardless of tumor type, are significantly associated with having upregulation of CTLA4 in RNA (far left-hand side) [as denoted with the red dots signifying upregulation of the IRM in RNA (see upper part of the panel for dots)]. Further, APC mutations are significantly associated with downregulation of TIM3 in RNA (far right-hand side) [as denoted with the blue dots signifying downregulation of the IRM in RNA (see upper part of the panel for dots)]. (See also Supplementary Table S4, available at <https://doi.org/10.1016/j.esmooop.2022.100396>.) Asterisks ( $n = 6$ ) were confirmed significant associations in an independent set of 2739 clinical cases from NantHealth. P value was adjusted based on the multiple hypothesis correction (false-discovery rate).

IRM may theoretically be an additional marker to genetic markers of immunotherapy response and resistance such as TMB, HLA, STK11, and B2M mutations.

Genes associated with DNA damage repair are under study in several solid tumors as potential new partners for immunotherapy; specifically, PARP inhibition can lead to upregulation of PD-L1 via inactivation of GSK3 $\beta$ , which may lend itself to targeting with combinations such as olaparib with pembrolizumab.<sup>20</sup> This combination is undergoing trial now in patients with advanced melanoma harboring mutations in various DNA damage repair genes (NCT04633902) as well as in patients with metastatic pancreatic ductal adenocarcinoma harboring similar DNA damage repair genes (NCT04666740). The same combination has shown benefit in docetaxel-pretreated patients with metastatic castration-resistant prostate cancer and extensive stage small-cell lung cancer.<sup>46,47</sup>

There are several limitations to this study, mostly based on the fact that the databases used are not clinically curated hence more comprehensive clinical correlations could not be made. Even so, it is apparent that incorporating NGS cancer driver gene panels in the care of patients with cancer is becoming more prevalent, and the findings presented here support the use of driver gene mutation status in selection of patients for treatments with/trials of, for example, anti-PD-1 therapies. Driver mutation-positive tumors are often characterized as TMB-low and consequently not treated with checkpoint inhibitors. With regard to PD-L1 expression, some of the associations found might not be only correlations, but related to the integral process of PD-L1 activation; more specifically, within the various transcription factors that may affect PD-L1 expression such as HIF-1, STAT3, NF- $\kappa$ B, and AP-1.<sup>48-51</sup> Further, the PI3KA, EGFR, and MAPK pathways and other gene products appear to influence PD-L1 expression, which was demonstrated within this dataset.<sup>52-55</sup> Within our dataset, *KRAS* mutations were associated with downregulation of PD-L1/2; however, other groups have found within NSCLC that *KRAS* mutations caused upregulation of PD-L1 and that *RAS* mutations stabilize PD-L1; the clinical significance of these findings is unclear.<sup>56,57</sup> These data support the notion that trials combining various targeted therapies and IRM inhibitors have biologic rationale and may have positive clinical impact.

It is unclear if tumors that express the various IRMs and targeting said IRM will have clinical benefit for patients; this research serves as the foundation for further prospective studies rationally combining various targeted therapies with developing IRM targeting agents.

We demonstrate specific mutations that are associated with IRM gene expression such that a rationale for checkpoint inhibitor treatment exists. While currently there may not exist therapies targeting some of the specific hotspot mutations or IRMs described here, efforts to identify such therapies are underway.<sup>58</sup> Our data support the merit of continued development of therapies directed to these targets with the goal of designing clinical trials of personalized combination therapies.

## FUNDING

This work was funded in part by the Joan and Irwin Jacobs Fund, and by National Cancer Institute grant P30 CA023100 (RK). Special thanks to Patricia Spilman for writing support.

## DISCLOSURE

JJA and AG have no disclosures. CWS, SV, AP, and SKR are employees of NantHealth. SK consults for Foundation Medicine; reports speaker's fee from Roche, and research grant from ACT Genomics, Sysmex, Konica Minolta, and OmniSeq. RK has research funding from Incyte, Genentech, Merck Serono, Pfizer, Sequenom, Foundation Medicine, Guardant Health, Grifols, Boehringer Ingelheim, and Konica Minolta, as well as receiving consultant and/or speaker fees from LOXO, X-Biotech, Actuate Therapeutics, Genentech, Pfizer, Roche, and NeoMed. RK has an equity interest in IDbyDNA and CureMatch, Inc and is a board member of CureMatch and CureMetrix and a co-founder of CureMatch.

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