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Peer Review File

53BP1 loss elicits cGAS-STING-dependent antitumor immunity in ovarian and pancreatic cancer

Editorial note: This manuscript has been previously reviewed at another journal that is not operating a transparent peer review scheme. This document only contains reviewer comments and rebuttal letters for versions considered at *Nature Communications*.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This study paves the way for the inclusion of Trp53bp1 as a predictive biomarker in clinical trials of ICB in BRCA patients with cancer. This study is of high significance for scientists and clinicians with an interest in personalised medicine of BRCA patients. The authors have addressed all my comments adequately. Appropriate controls have been included and additional evidence to further support the conclusions of the study have been provided. I am happy to support publication of the revised manuscript, pending minor corrections related to the referencing to figure panels:

Lines 196-98: The authors stated "Additionally, our interrogation of the TISIDB demonstrated decreased TP53BP1 expression correlated with increased activated CD8+ T-cell infiltration (Fig. 1e, f)". I believe the correct reference to be to Supplementary Fig 1d.

Lines 381-382: The authors stated "The Brca1 Trp53bp1 DKO cells had significantly more micronuclei (Supplementary Fig. 5a, b)". I believe the correct reference to be to Supplementary Fig 5c, d.

Lines 435-36: The authors stated "and NKp46+ NK cell (Fig. 6d) infiltration. In addition, low 53BP1 expression correlated with increased PD-L1 expression (Fig. 6c)." The references to figure 6 panels are the wrong way round.

Reviewer #2 (Remarks to the Author):

In this revised manuscript, Sun et al. have addressed some of the comments we raised. However, fundamentally, whether or not checkpoint inhibition will play a role in PARP

inhibitor resistant ovarian cancer is certainly not an argument supported by the data in this manuscript.

The authors cite the TOPACIO/KEYNOTE-163 trial - which was a single arm phase I/II trial with a lead-in and testing Niraparib in combination with Pembrolizumab - as supporting evidence for their hypothesis. However, it is important to note several factors:

1) This trial is a single arm trial and therefore it is not adequate to use data from this trial and compare it to other treatment modalities. There are plenty of confounders. For instance one could make the argument that anti-tumor activity is attributed to PARP inhibition alone and there is evidence in the ovarian cancer field that PARP inhibitors alone have a strong signal in the HRD subset.

2) This trial combined ICB with PARPi and therefore it did NOT address whether PARPi resistant ovarian cancer would be responsive to ICB if they lost 53BP1. Granted some of the responders were platinum resistant or refractory. To make such a claim using the trial data, one would need to investigate this in post PARPi resistance population which is not what this trial attempted to address.

3) To make the claim that loss of 53bp1 is a predictive biomarker for response to ICB in the PARPi resistant setting then it would be important to perform a tissue based analysis in this population, or at least samples on the trial for those patients who have responded vs not to show differences in 53bp1.

More importantly, a key concern is the use of highly selected pre-clinical models that raises concerns about the clinical translational potential of this work. Depending on the experimental context, ID8 has been shown to exhibit some response to ICB. It would be mode convincing if some of the key findings were reproduced in additional models especially ones that are known to be strongly immune resistant.

The authors should comment on the potential source of IFN-g if not T-cells. Also the focus on type II IFN is confusing given the well known role for STING to promote type I IFN. It

might be worth considering ruling out a contribution for type I IFN by using IFNAR1 blocking antibodies.

Unless I missed it the authors did not demonstrate that endogenous cGAS stains micronuclei in these tumor models which is critical. Furthermore, there was no quantification of cGAMP in cells or tumors as initially proposed. This is a critical point to rule out non-canonical STING activation which has been previously shown to be cGAS independent (PMID: 30193098). At the very least, the authors should perform trp53bp1 Cgas DKO to demonstrate that the effect is determined through the pathway they propose.

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We thank the reviewer for the generous appreciation of our study.

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We thank the reviewer for pointing out these mistakes, we have made the necessary corrections in the revised manuscript.

Reviewer #2

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1) This trial is a single arm trial and therefore it is not adequate to use data from this trial and compare it to other treatment modalities. There are plenty of confounders. For instance one could make the argument that anti-tumor activity is attributed to PARP inhibition alone and there is evidence in the ovarian cancer field that PARP inhibitors alone have a strong signal in the HRD subset.

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We appreciate the reviewer's comment but would like to clarify that our argument for ovarian cancer is that loss of 53BP1, which has been shown to be a frequent alteration and cause of PARPi resistance in BRCA1-mutated tumors, may be overcome by ICB in the setting of BRCA-mutated tumors. We acknowledge that, unfortunately, there have been no immunotherapy trials involving patients with PARPi resistant BRCA-mutated ovarian tumors to directly test this hypothesis. However, while we agree with the reviewer that the TOPACIO study did not address whether PARPi resistant ovarian cancers would be responsive to ICB, it is important to underscore that:

i) 75% of patients in TOPACIO were platinum resistant/refractory and given that it is well established that there is significant cross-resistance between platinum and PARPi in HGSOC (due to overlapping mechanisms of resistance to platinum and PARPi in this disease) the majority of these patients are also expected to be PARPi resistant.

ii) There were 11 patients with BRCA-mutated tumors enrolled in TOPACIO and 2 (18%) of these patients responded to niraparib/pembrolizumab, one with a complete response (CR), and the other with ongoing, durable (>18 months) partial response (PR). These responses are notably better than expected in this patient population for PARPi or PD-1 inhibitor monotherapy suggesting that addition of ICB to PARPi may have overcome platinum (and concurrent PARPi) resistance in these BRCA-mutated tumors.

iii) There are no available samples from the TOPACIO trial to perform any additional analyses, and even if there were, the number of patients with BRCA-mutated tumors was too small for any meaningful statistical comparison in 53BP1 levels.

Beyond the TOPACIO study, we would like to also highlight another trial, the JAVELIN PARP Medley Nonrandomized Controlled Trial which showed prolonged duration of response in patients with BRCA-mutated ovarian cancer with the combination of PARPi talazoparib and PD-L1 inhibitor avelumab (PMID: 36394849). Notably, in that study, 55% of patients with BRCA-mutated ovarian cancer were alive and progression free at 18 months suggesting notably improved durability of response compared with that seen with PARPi monotherapy in similar populations (such as in the SOLO3 (PMID: 32073956) and NRG-GY004 (PMID: 35290101) studies). This suggests that besides overcoming PARPi resistance, adding ICB to PARPi may also prevent PARPi resistance which, in the setting of BRCA-mutated tumors, may reflect the fact that development of acquired resistance due to 53BP1 loss is effectively suppressed by addition of ICB.

Taken together, we would like to point out that there have been no immunotherapy trials involving patients with PARPi resistant BRCA-mutated ovarian tumors to directly test whether ICB may overcome resistance due to 53BP1 loss. However, available clinical data from patients with BRCA-mutated tumors

treated with PARPi/ICB combinations are indeed consistent with the hypothesis that addition of ICB may prevent as well as overcome resistance due to 53BP1 loss.

More importantly, a key concern is the use of highly selected pre-clinical models that raises concerns about the clinical translational potential of this work. Depending on the experimental context, ID8 has been shown to exhibit some response to ICB. It would be mode convincing if some of the key findings were reproduced in additional models especially ones that are known to be strongly immune resistant.

We agree with the reviewer about the importance of replicating our findings in additional models and indeed have reproduced our findings in a mouse pancreatic cancer model (KPC), using both orthotopic and subcutaneous tumor engraftment models, which are resistant to immunotherapy (PMID: 32946773). Almost all the data has been generated with KPC (pancreatic) and ID8 (ovarian) representing two immunotherapy resistant malignancies.

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*We have clarified the text. To understand how Trp53bp1 depletion might impact biological systems and pathways, we performed RNA-sequencing and pathway analysis which demonstrated a robust increase in the interferon gamma response, inflammatory response, interferon alpha response… (**Fig. 1i; Supplementary Fig. 1i**) suggesting that Trp53bp1 depletion results in an inflamed state secondary to the activation of multiple pathways, in addition to the cGAS-STING pathway which further assess in the manuscript. We hypothesize that the increased interferon gamma response is secondary to the observed increased T-cell infiltration. Indeed, in **Figure 3**, we observed increased IFN-g as a marker of T-cell activation in Trp53bp1 KO tumors. Regarding type I IFN, in **Figure 4g**, we observe decreased expression Ifnb1 upon Sting depletion in a Trp53bp1 KO background. Indeed, depletion of Sting in a Trp53bp1 KO background abrogated the tumor growth inhibition seen upon Trp53bp1 depletion alone, indirectly suggesting a contribution of type I IFN. IFN-gamma is a common marker for T cell activation and killing effect. We demonstrated that more T cells were activated in 53BP1 KO tumors by IFN-g. Type II IFN is a final marker to show immune activation. Here we are not attempting to define the contribution of type I or type II IFN.*

Unless I missed it the authors did not demonstrate that endogenous cGAS stains micronuclei in these tumor models which is critical. Furthermore, there was no quantification of cGAMP in cells or tumors as initially proposed. This is a critical point to rule out non-canonical STING activation which has been previously shown to be cGAS independent (PMID: 30193098). At the very least, the authors should perform trp53bp1 Cgas DKO to demonstrate that the effect is determined through the pathway they propose.

*We thank the reviewer for these comments regarding cGAS. First, we have now performed immunofluorescence demonstrating that the increased micronuclei produced upon Trp53bp1 depletion are cGAS-bound (**Figure 4c**). Second, we have now performed cGAMP ELISA demonstrating that*

*Trp53bp1 depletion increases cGAMP production (**Figure 4d, e**). Third, we have now knocked out cGAS in our ID8 and KPC Trp53bp1 KO cells (**Supplementary Figure 4e, f**). Confirming the anti-tumor effect of TP53BP1 loss is mediated by cGAS-STING pathway activation, loss of Cgas abrogated phosphorylation of Sting and Tbk1 (**Supplementary Figure 4e, f**). Consistently, loss of Cgas abrogated the increased mRNA expression of a panel of inflammatory genes seen upon Trp53bp1 loss (**Supplementary Figure 4g, h**).*

REVIEWERS' COMMENTS

Reviewer #2 (Remarks to the Author):

I would like to thank the reviewers for their thoughtful responses and additional experimental work bolstering the role of cGAS in their observed phenotypes. I would support publication of this work provided the authors carefully re-word and qualify the text to acknowledge the caveats associated with the clinical trials conducted to date as well as the challenges as well as promises of translating the current work.

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I would like to thank the reviewers for their thoughtful responses and additional experimental work bolstering the role of cGAS in their observed phenotypes. I would support publication of this work provided the authors carefully re-word and qualify the text to acknowledge the caveats associated with the clinical trials conducted to date as well as the challenges as well as promises of translating the current work.

We thank the reviewer for the suggestion. We added the sentences in the manuscript (page 19): "Indeed, analysis of the IMvigor210 trial, demonstrated that lower TP53BP1 expression correlated with enhanced response to ICB and improved patient outcome. Importantly, despite 53BP1 being a member of the Shieldin complex, expression of other DSB end-protection factors did not associate with increased immune infiltration or improvement in overall survival, and did not predict response to ICB, reinforcing the importance of 53BP1 as the primary counterpart of BRCA1 and its critical role in DSB repair. Unfortunately, there have been no immunotherapy trials involving patients with PARPi resistant BRCA-mutated ovarian tumors to directly test our hypothesis. However, our results indicate that patients with PARPi-resistant BRCA1-mutated 53BP1 loss cancers might benefit from a combination of PARPi and immunotherapy. This indicates the feasibility of initiating clinical trials to explore the use of immunotherapy in patients with PARPi resistant BRCA-mutated and 53BP1 loss ovarian tumors. This line of investigation not only holds promise for offering new hope to patients with these types of tumors but also contributes to expanding therapeutic strategies for ovarian cancer. "