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Editorial note: This document refers to unpublished data that have now been published under the following reference:
Seo, E.S., et al. Response-adapted consolidation therapy strategy for patients with metastatic high-risk neuroblastoma: Results of the SMC NB-2014 study. *Pediatr Blood Cancer,* e31173 (2024).

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author): Expert in cancer genetics and genomics, germline variants, neuroblastoma, bioinformatics, and statistics

The manuscript entitled "Germline Functional Variants Contribute to Somatic Mutation and Outcomes in Neuroblastoma" by Seo and Lee et al presented a very interesting analysis using germline and somatic variants identified in neuroblastoma. By focusing on putatively functional germline variants (pFGVs) identified by population frequency filtering (<1%) and in-silico prediction on deleterious effect (Revel score >0.7, ClinVar P/LP, or LOF mutations), a positive correlation was identified in burden of germline variants and somatic mutations in their cohort of 125 Korean neuroblastoma patients at the Samsung Medical Center (SMC cohort). They also found that higher pFGV burden is associated with worse clinical outcome. Similar analysis was performed on ~200 NCI TARGET NBL samples with the attempt to validate the findings made in SMC cohort. A comparison to the adult TCGA data showed a different profile in adult cancer. The study can be of potential interest to biomedical community as the use of pFGV burden as a potential prognostic marker is a new concept that can be tremendous interest.
Major issues:
1) Positive correlation between germline variant burden and somatic mutation burden. This is a key finding of the paper and was in contrast with the negative but much significant findings in adult cancer published by Qing et al (Nat Commun. 2020; 11: 2438, Figure 2e, $r = -0.70$, $P = 0.017$). Given the correlation shown in Figure 1c is very weak and barely reached statistical significance($r=0.18$, $p=0.041$), more rigorous analysis needs to be performed to ensure that the result was not caused by sampling bias. One possibility is to perform downsampling on the synonymous variants (which is five times higher than pFGVs) shown in Figure 1d to the same count distribution as pFGVs to evaluate this possibility.
2) Higher coverage in tumor and normal samples can both lead to increased variant calls. Given the weak correlation, it is possible that sequencing coverage bias can lead to the weak positive correlation. The authors need to demonstrate that the increased somatic and germline mutation burden was not due to differences in coverage within the SMC cohort as well as in the comparison of healthy/cancer cohorts.
3) Replication of negative correlation using TARGET NBL. The negative correlation shown in Figure 2B appears to be driven by a few outlier samples with very high germline mutation burden. It should be noted that the 222 TARGET neuroblastoma samples are from patients with 9 ethnicity groups based on Supplementary Table S1 of Pugh et al, Nat. Gen. 2013. Yet the germline analysis, as documented in Methods, did not taken into account the ethnicity diversity of this cohort. Therefore, the germline variant count shown in Figure 2B likely represents those from non-Caucasian individual in TARGET NBL. It should be noted that the highest germline burden in TARGET NBL (150-200 mutations) also exceeded the germline burden identified in TCGA cohort (a much larger cohort filtered to retain only Caucasian cases, all <=150 mutations based on Figure 2c), raising concerns on the accuracy of germline count analysis presented in this study. Given the lack of clarify on consideration for diverse ethnicity in TARGET NBL, Figure 2B can not be viewed as a validation for findings made in the Korea cohort.
4) Figure 2d. The positive correlation between germline variant burden and somatic mutation burden in adult TCGA cohort replicates the previous published results in Figure 2 by Qing et al (Nat Commun. 2020; 11: 2438). However, Qing et al has specifically mentioned that the trend was driven by age-associated increase in somatic mutation burden in the following statement in their paper: "The sM burden showed a significant positive correlation with age (Beta = −0.018, P = 0.0030) but the gHFI variant burden did not

(Beta = 0.46, P = 0.91). This indicates that the strong negative correlation between gHFI and sM across age groups is primarily driven by the age associated increase in sM burden." Therefore, the authors need to evaluate whether the negative correlation is related to the low somatic mutation burden in neuroblastoma. Additionally, the correlation for TCGA presented in this study appears to be weaker compared to the results by Qing et al. which needs to be discussed in the manuscript.
5) CPG analysis was based on pFGVs, which is a non-standard approach as the community primarily focuses on using P/LP variants for enrichment analysis. Was the clinical outcome and enrichment compared to the healthy population driven by P/LP variants in CPG? A comparison is needed to clarify the role of pFGVs versus P/LP variants.
Minor points:
1. A comparison of somatic mutation burden in SMC with other published neuroblastoma data set is needed to ensure the accuracy of the data analysis.
2. Figure 2d. TARGET data set only includes the TARGET neuroblastoma based on the description in Methods. Please clarify in the label and figure legend.
3. Abstract—all descriptive without the statics to justify the importance.
4. Need to clarify the data source for CPG. The results shown in lines 122-123 indicated that 109 CPGs were analyzed when reference to Supplementary Table S1 which listed all 733 Cancer Gene Census from COSMIC and the authors need to show only the CPG genes in this table (presumably those labeled as Germline in second column).
5. Figure 1a. Population filtering. KRGDB 1100 <1% was used for filtering SMC cohort but not the TARGET/TCGA cohort. This can cause potential problem with the rare variants in Korean/Asian patients involved in TARGET/TCGA as one variant may be considered as pFGVs in one cohort but non-pFGV in the other. Please evaluate this scenario.
6. Supplementary Table S1: Gene symbols for SEPT5, SEPT6 and SPET9 are in date format in excel sheet

Reviewer #2 (Remarks to the Author): Expert in neuroblastoma genomics and clinical research

Review of Germline Functional Variants Contribute to Somatic Mutation and Outcomes in Neuroblastoma

Seo et al perfomed germline whole exome sequencing of a cohort of 125 patients with neuroblastoma from both HR and IR/LR risk groups and showed that the burden of putative functional germline variants (predicted LOF and missense mutations - termed pFGVs by Seo et al) is associated with the somatic mutation burden in neuroblastoma as well as having a prognostic impact in neuroblastoma.

They then go on to demonstrate that pFGVs in cancer predisposing genes (CPGs) are enriched in neuroblastoma (both using their own cohort as well as TARGET-NBL; comparing with KOREA1K and TCGA, respectively) and then demonstrate that pFGVs (specially when additional filtering according to the ACMG clinical criteria for likely pathogenic and pathogenic (LP/P)) alter prognosis in neuroblastoma — predominantly in MYCN-negative cases.

The paper is in general well structured and easy to read, and in my mind especially that last results where Seo et al demonstrate that presence of LP/P-variants in CPGs affects overall survival are quite interesting, also in a clinical context. I do, however have some questions regarding methodology that I feel needs to be addressed:

* In their testing of the burden of germline variants and its association with clinical variables they dichotomise the germline variant burden — this is not statistically sound practice, they should test the germline variant burden as a continuous variable here.
* In general one needs to be wary of potential differences in ethnicity when doing genetic comparisons between cohorts, have the authors perfomed any analysis to this end? (e.g. PCA-plots of common SNPs) if not, this needs to be done and included in the paper.
* The methods section needs to be expanded with more detail. What exome enrichment kit was used? They also state that some of the tumor samples (from where somatic mutations were called) was from FFPE tissue, I would be very vary of comparing (somatic) mutational burdens between fresh frozen samples and FFPE samples — it needs to be acknowledged which SMC samples had tumor DNA extracted from FFPE tissue samples.
* To what depth was the germline and tumor samples from the SMC cohort sequenced?
* Re-calling the TARGET-NBL data was in my mind absolutely the right thing to do, and they also correctly note (and mitigate) the known issues with the somatic mutations in the TARGET-NBL dataset. Furthermore, how does the variant calling of the TCGA samples differ from the TARGET-samples? Ideally they should be processed through the sample variant calling pipeline. At the very least, the differences needs to be clearly stated.
* The code used needs to be publicly available on GitHub or FigShare.
* In Figure 1C: there are some clear outliers in somatic mutational burden, are these variant calls derived from FFPE tissues? Did the patients have germline mutations in CPGs that are known to give an increased number of somatic mutations?
* Figure 3: Need to treat the Germline variant burden as a continuous variable and not dichotomise in to high and low (causes loss of information)
* Figure 4: some cases with germline mutations in genes known to cause an increased mutational load - did they perform further analysis with this subgroup removed?
* Figure 4: Were these results communicated back to patients and their families? At least for the patients with mutations in mismatch repair genes and TP53 it would be significant for them to know they have an increased risk of cancer which would give them the option to participate in screening programs etc.
* Figure 5D: also dichotomisation — needs to be re-analyzed using the Germline Variant burden as a continuous measure.
* Figure 6: this is really interesting and to me the key finding!
* Extended Data Fig.1 : Why are they mixing Pearsons r with Spearman's rank correlation coefficient? (And why log10-transform data when using a non-parametric correlation measure such as Spearman's?)
* Extended Data Fig.5: This is really interesting and could be very useful in a clinical setting in a short timeframe. The authors should perform the same analysis also on the TARGET-NBL cohort to se if it holds.

To summarise: I find the paper and its findings interesting and of potential clinical importance but my (mainly methodological) points above needs to be addressed.

Reviewer #3 (Remarks to the Author): Expert in neuroblastoma genetics, predisposition, and therapy

The authors performed whole-exome sequencing of 125 patients with neuroblastoma from South Korea to study the role of putatively functional germline variants (pFGVs) in neuroblastoma pathogenesis. This study focused on 109 cancer predisposition genes (CPGs) listed in the Cancer Gene Census (CGC) from the Catalogue Of Somatic Mutations In Cancer (COSMIC) database (page 5). The CGC lists 738 genes, so presumably the 109 genes included in this study were chosen because they were CPGs, many of which (80) are possibly involved in DNA stability and repair mechanisms. They found a direct correlation between pFGVs and somatic mutations in tumors, as well as with patient outcome (higher pFGVs correlated with worse outcome). Similar results were seen in a separate neuroblastoma cohort, but not seen when analyzing an adult cancer cohort. They conclude that the combination of germline and clinical risk factors improves survival predictions.

The connection between the burden of pFGVs and the development of neuroblastoma in this study is conjecture at best and hardly actionable, as there are a number of different CPGs affected, and none have a direct association with neuroblastoma predisposition. The burden appears to be a single germline change in most cases (34 of 39 according to Figure 4), two mutations in 5 other cases, and most are missense mutations. It is difficult to understand how a mutation in any one of the genes listed selectively increase the risk of neuroblastoma. The pFGV burden is not explored in much detail, and according to Figure 4 seems to involve only a single gene in most cases. Moreover, it is not clear if they are proposing the use of pFGV burden only as a prognostic marker or also as an insight into cancer predisposition. The top genes involved include FAT1, MLH2, MSH2, BRCA2, MAX, and TP53, most of which are known to be associated with DNA instability or repair, so a germline mutation, especially a truncating mutation, could be a contributing factor to increased somatic mutations in tumors. In addition, the use of pFGV "burden" as a prognostic marker is hard to understand or study, when there are dozens of serum biomarkers, tumor expression profiles, or other predictive markers and algorithms that have shown similar or stronger predictors of outcome.

Germline mutations in a few genes predispose to development of neuroblastoma, such as ALK and PHOX2B, with high penetrance (~50% each). There are a few other genes associated with syndromes that also predispose to neuroblastoma, such as CDKN1C mutations in Beckwith-Wiedemann syndrome and KRAS in Costello syndrome, in which the penetrance of neuroblastoma is lower (1-5%). Finally, there have been dozens of genes identified by GWAS studies that were called neuroblastoma "susceptibility"

or “predisposition” genes, but most genes implicated by GWAS have been one-off observations with weak effects, and some of the SNPs are near but not even in the gene, so they are not really actionable as CPGs without further investigation.

There are a large number of clinical, laboratory, genetic, genomic, expression, radiographic, pathologic, and other predictive markers of neuroblastoma prognosis. Furthermore, there have been somatic genetic studies identifying single genes (e.g., MYCN, ALK) that have prognostic value, but there have also been dozens or hundreds of reports of expression studies of single genes, small groups, and larger panels that predict outcome in neuroblastoma, but essentially none of these have stood the test of time or become implemented in national or international cooperative group studies. Indeed, what is really needed for neuroblastoma as well as other pediatric tumors is more effective, less toxic therapy, not more prognostic markers or predictive algorithms.

The patient cohort on which they focused was 125 neuroblastoma patients, but they do not specify over what period of time these patients were diagnosed, how they were selected, or if they were representative. Given that about half were high-risk and half were low or intermediate risk, they are presumably representative, but it would be helpful to clarify this. Also, they mention the “clinical” risk factors of age, stage, and MYCN status (page 6), but current risk prediction algorithms in the US, Canada, Europe, and Japan use more complex algorithms. Figure 4 does list age, sex, stage, risk group, path, and MYCN status, so presumably they used all of these as their “clinical” risk markers, so this should be clarified in the text. Details of the patient characteristics are shown in extended table 1, but they should indicate whether the breakdown of different markers are similar to an unselected series of patients.

My other concerns about this manuscript are that the effect sizes and p-values are almost all very weak. Many of the analyses presented had marginally significant p-values, such as 0.018, 0.032, 0.024, etc., and none of these would survive a Bonferroni correction. They did apply this correction to the added predictive value of germline risk factors in neuroblastoma (page 6 and Figure 7), but not to other statistical analyses.

Responses to reviewer's comments on the manuscript submitted by Seo et al., "Germline Functional Variants Contribute to Somatic Mutation and Outcomes in Neuroblastoma" (Manuscript ID: NCOMMS-23-27293-T)

We extend our gratitude to the reviewers for their comprehensive review and valuable insights on our manuscript. Their critical observations and constructive feedback have been instrumental in enhancing the quality of our work. We have carefully considered their comments and accordingly revised our manuscript, aligning it closely with their recommendations.

In the attached document, we respond to each of the reviewers' comments. For clarity, the reviewers' comments are highlighted in bold, with our responses following in blue. We have explained the rationale behind the revisions made and, in cases of differing perspectives, have provided our reasoning. Major changes in the revised manuscript are indicated by page and line numbers and are highlighted for ease of reference. Where specific suggestions from reviewers were not adopted, we have offered detailed explanations to clarify our position.

We hope that these revisions meet the expectations of the review panel and look forward to further suggestions or feedback.

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author): Expert in cancer genetics and genomics, germline variants, neuroblastoma, bioinformatics, and statistics

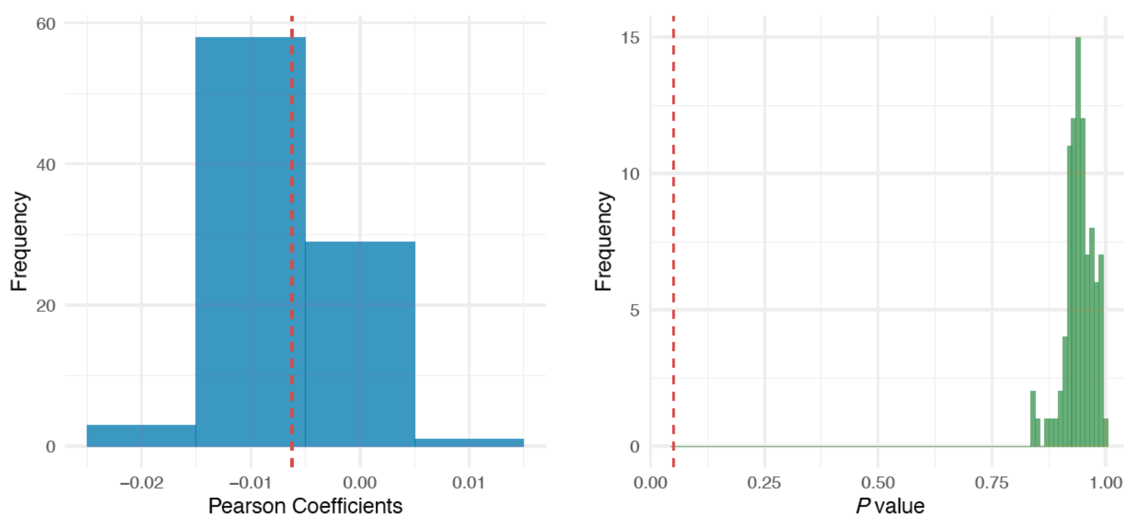
General comment: The manuscript entitled "Germline Functional Variants Contribute to Somatic Mutation and Outcomes in Neuroblastoma" by Seo and Lee et al presented a very interesting analysis using germline and somatic variants identified in neuroblastoma. By focusing on putatively functional germline variants (pFGVs) identified by population frequency filtering (<1%) and in-silico prediction on deleterious effect (Revel score >0.7, ClinVar P/LP, or LOF mutations), a positive correlation was identified in burden of germline variants and somatic mutations in their cohort of 125 Korean neuroblastoma patients at the Samsung Medical Center (SMC cohort). They also found that higher pFGV burden is associated with worse clinical outcome. Similar analysis was performed on ~200 NCI TARGET NBL samples with the attempt to validate the findings made in SMC cohort. A comparison to the adult TCGA data showed a different profile in adult cancer. The study can be of potential interest to biomedical community as the use of pFGV burden as a potential prognostic marker is a new concept that can be tremendous interest.

Response: We greatly appreciate the reviewer's positive comments. We have carefully revised the manuscript according to the reviewer's insightful comments. We have addressed each point below and we feel that the manuscript is greatly improved as a result.

Major issues:

Comments 1-1: Positive correlation between germline variant burden and somatic mutation burden. This is a key finding of the paper and was in contrast with the negative but much significant findings in adult cancer published by Qing et al (Nat Commun. 2020; 11: 2438, Figure 2e, $r = -0.70$, $P = 0.017$). Given the correlation shown in Figure 1c is very weak and barely reached statistical significance ($r=0.18$, $p=0.041$), more rigorous analysis needs to be performed **to ensure that the result was not caused by sampling bias**. One possibility is to perform downsampling on the synonymous variants (which is five times higher than pFGVs) shown in Figure 1d to the same count distribution as pFGVs to evaluate this possibility.

Response 1-1: We appreciate the reviewer's constructive comment and think this is a great suggestion. To this end, we undertook a systematic downsampling analysis of the synonymous variants, given their notably larger volume compared to pFGVs. Specifically, we incrementally downsampled the synonymous variants, starting from 10% up to 100% in 1% steps. For each downsampling fraction, we recalculated the correlation between the number of rare synonymous germline variant and the somatic mutational burden. Below this paragraph, we show both correlation coefficients (rho) and corresponding p-values of this analysis. As we can see from this figure, this analysis provides a clearer understanding of the non-significant relationship between synonymous variants and somatic mutations.



We have incorporated this analysis into the revised results and methods sections as detailed below:

Page 4, lines 108−110: "*These findings persisted when we implemented a down-sampling analysis, addressing potential biases due to the disproportionate volume of synonymous variants in relation to pFGVs (Extended Data Fig. 1b,c).*"

Page 13, lines 365−367: "*In the downsampling analysis of synonymous variants, we incrementally reduced their count in 1% increments, starting from 10% and progressing to 100%. At each step of this process, the correlation between the number of rare synonymous germline variants and the total somatic mutation burden was recalculated.*"

Additionally, Extended Data Fig. 1b and 1c have been updated.

Comments 1-2: Higher coverage in tumor and normal samples can both lead to increased variant calls. Given the weak correlation, it is possible that sequencing coverage bias can lead to the weak positive correlation. The authors need to demonstrate that the increased somatic and germline mutation burden was not due to differences in coverage within the SMC cohort as well as in the comparison of healthy/cancer cohorts.

Response 1-2: We appreciate the reviewer's concern regarding the potential bias introduced by sequencing coverage. Indeed, the accuracy of mutation or variant counts can be influenced by sequencing depth.[1] We investigated the correlation between sequencing coverage and mutation/variants counts in the SMC cohort. Although we found some possibility that germline variant burden was influenced by sequencing coverage of normal, it was not statistically significant (Spearman' $\rho = 0.17$, $P = 0.06$). Given that sequencing depth did not meet the normality assumption (Shapiro-Wilk test $P = 0.004$), we used the Spearman correlation. Importantly, somatic mutational burden was not affected by tumor sequencing coverage (Spearman' $\rho = -0.07$, $P = 0.435$), and germline sequencing depth also showed no association with somatic mutational burden (Spearman' $\rho = 0.07$, $P = 0.417$). These findings underscore that, while germline variant calls could potentially be swayed by sequencing

depth, the correlation between germline variant burden and somatic mutational burden likely stems from inherent biological factors rather than sequencing depth alone. To rigorously address the coverage influence, we employed a multivariable linear regression model, factoring in sequencing depth in tumor and germline, and clinical risk group. Even after adjusting for these parameters, germline variant burden maintained a significant positive correlation with somatic mutational burden (Beta = 0.01, *P* = 0.045). In our analysis of the TARGET NBL, comprised solely of high-risk patients, the model was adjusted for sequencing depth in both tumor and germline, as well as reported race. Again, a significant association between germline variant burden and somatic mutational burden emerged (Beta = 0.01, *P* = 0.016).

In light of the insightful comments made by the reviewer, we have amended our manuscript to incorporate the following analytical details:

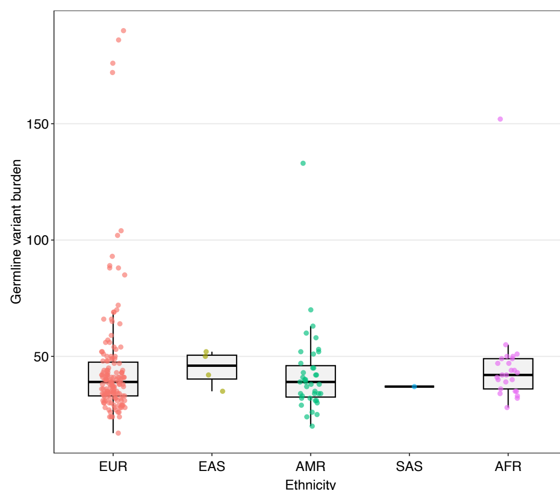
Page 4−5, lines 119−123: "*To account for potential confounding factors, we employed a multivariable regression analysis. In analysis of the SMC cohort, after adjusting for median sequencing depth in both tumor and germline and the clinical risk, the somatic mutational burden continued to show a significant positive association with germline variant burden (β = 0.01, P = 0.045). This association was consistent among TARGET neuroblastoma patients, even when adjustments were made for race and median sequencing depth in both tumor and germline (β = 0.01, P = 0.016).*"

Comments 1-3: Replication of positive correlation using TARGET NBL. The positive correlation shown in Figure 2B appears to be driven by a few outlier samples with very high germline mutation burden. It should be noted that the 222 TARGET neuroblastoma samples are from patients with 9 ethnicity groups based on Supplementary Table S1 of Pugh et al, Nat. Gen. 2013. Yet the germline analysis, as documented in Methods, did not taken into account the ethnicity diversity of this cohort. Therefore, the germline variant count shown in Figure 2B likely represents those from non-Caucasian individual in TARGET NBL. It should be noted that the highest germline burden in TARGET NBL (150-200 mutations) also exceeded the germline burden identified in TCGA cohort (a much larger cohort filtered to retain only Caucasian cases, all <=150 mutations based on Figure 2c), raising concerns on the accuracy of germline count analysis presented in this study. Given the lack of clarify on consideration for diverse ethnicity in TARGET NBL, Figure 2B can not be viewed as a validation for findings made in the Korea cohort.

Response 1-3: We sincerely thank the reviewer for bringing up an issue that we find to be both of utmost importance and enormously troubling. We have made minor amendments to the reviewer's comment for accuracy and clarity, with changes highlighted in red. While we utilized the Spearman correlation for its robustness against outliers, we recognize the necessity of incorporating ethnicity into our analytical framework, particularly given the heterogeneity of the TARGET neuroblastoma cohort. This offers us an opportunity to provide a more detailed

explanation of our analysis. Please bear with our lengthy of elaboration.

- **Ethnic Diversity in Analysis**: During our research, especially while validating our results with the TARGET NBL cohort, the handling of ethnic variation posed significant challenges. In our analysis of the 220 TARGET patients, the ethnic breakdown is as follows: 160 White, 29 Black or African American, 24 Unknown, 1 American Indian, 3 Asian, and 3 Native Hawaiian. The data presented challenges in sub-categorizing ethnicities. For instance, the Asian category lacked specifics like East Asian or South Asian descent, making it challenging to apply ethnic matched variant filtering using population allele frequency. To address this, we have now incorporated the EthSEQ package (v 3.0.2), which uses reference genotype data from 10,000 exonic SNPs to more accurately determine ethnicity (Extended Data Fig. 8). Subsequent germline variant recalculations in the TARGET dataset now utilize an ethnicity-matched reference, ensuring a more accurate representation of variant burden. It is now evident that germline variant burden does not significantly differ across ethnicities within the TARGET cohort. These adjustments have yielded revised all statistics and figures in the manuscript. We appreciate this opportunity to refine our analysis, thereby strengthening the validity of our findings.



- **Disparity with TCGA Cohort**: The reviewer also noticed that germline burden in TARGET NBL exceeded that in the TCGA. The differences in germline burden observed between the TARGET NBL and TCGA cohorts necessitate additional clarification. It's essential to mention that samples with a higher count of germline variants were predominantly classified as the white race (see above figure). This disparity cannot be solely ascribed to ethnic diversity but also to the differences in variant filtering approaches between cohorts. For the TARGET NBL analysis, we implemented a less stringent variant allele frequency cutoff of 0.2 compared to the 0.3 used in the TCGA. This adjustment was made in consideration of the TARGET's lower sequencing depth (median coverage at 49X versus TCGA's reported average of 100X) and followed the literature which addressed TARGET data. Such methodological differences, beyond ethnic diversity, could contribute to the increased variant counts observed in TARGET. These methodological discrepancies suggest that caution should be exercised when making direct comparisons between cohorts. Therefore, our analysis focused within each cohort and we acknowledge that inter-cohort comparisons may require a careful

approach. We have now added this limitation in the discussion section.

Page 9, lines 255−258: *"Additionally, the total count of germline variants identified could have been influenced by the specific experimental design and the variant filtering processes applied, which varied across cohorts. Therefore, the germline variant burden must be interpreted with caution at an individual level. Such variance in methodology also renders direct comparisons between cohorts challenging."*

We also have included additional analysis that excludes outliers identified by a Z-score threshold of 3, which consistently supported our conclusions to mitigate the reviewer's concern regarding outlier influence, (as detailed in the updated Extended Data Fig. 2b).

Page 4, lines 115−118: *"This correlation persisted in patients without pFGVs in DDR genes (Spearman's ρ = 0.33; P < 0.001; Extended Data Fig. 2a), as well as in analyses that excluded outliers identified using a Z-score threshold of 3 (Spearman's ρ = 0.24; P < 0.001; Extended Data Fig. 2b)."*

Lastly, we have refined our survival analysis within the TARGET cohort by adjusting for ethnicity in relation to the burden of germline variants.

Page 5, lines 144−146: *"After adjusting for ethnicity and MYCN status, the impact of a higher germline variant burden remained statistically significant (adjusted HR, 1.70; 95% CI, 1.19−2.42; P = 0.003)."*

Comments 1-4: Figure 2d. The negative correlation between germline variant burden and somatic mutation burden in adult TCGA cohort replicates the previous published results in Figure 2 by Qing et al (Nat Commun. 2020; 11: 2438). However, Qing et al has specifically mentioned that the trend was driven by age-associated increase in somatic mutation burden in the following statement in their paper: "The sM burden showed a significant positive correlation with age (Beta = −0.018, *P* = 0.003) but the gHFI variant burden did not (Beta = 0.46, *P* = 0.91). This indicates that the strong negative correlation between gHFI and sM across age groups is primarily driven by the age associated increase in sM burden." Therefore, the authors need to evaluate whether the positive correlation is related to the low somatic mutation burden in neuroblastoma. Additionally, the correlation for TCGA presented in this study appears to be weaker compared to the results by Qing et al. which needs to be discussed in the manuscript.

Response 1-4: We appreciate the reviewer's detailed feedback and their reference to the work by Qing et al. Qing's research was a study that we considered highly valuable for reference. We understand the essence behind the reviewer's concerns, though we recognize that there might have been some confusion in the reviewer's comment. In order to respond accurately and provide clarity, we have made slight amendments in the reviewer's comment, with the changed words highlighted in red.

Qing et al.'s research logic can be succinctly summarized as follows: with an increase in age, there's no significant decline in the germline variant burden within cancer genes coupled with a significant rise in the somatic mutational burden. Consequently, the observed negative correlation between germline variants and somatic mutations is predominantly influenced by age.

- **Factors affecting somatic mutation in neuroblastoma:** In stark contrast, in neuroblastoma, age doesn't exert a significant impact on either the somatic mutations or the germline variant burden (r = 0.06, P = 0.504). Thus, age-adjustment for neuroblastoma is unnecessary. However, other clinical factors such as *MYCN* amplification and stage are associated with somatic mutational burden in neuroblastoma. These factors could be summarized as clinical risk group. After adjusting clinical risk group, germline rare burden maintained its significant association with somatic mutational burden (Beta = 0.01, P = 0.023).

	Odds ratio	*P* value
Risk		
Low	Ref	Ref
Intermediate	0.04	0.660
High	0.30	< 0.001
Germline variant burden	0.01	0.023

In the revised manuscript, we further adjusted clinical risk group as well as sequencing depth and the results were as follows: *In the SMC cohort, even after adjusting for median sequencing depth in both tumor and germline, as well as for the clinical risk group, the somatic mutational burden maintained a significant positive association with germline variant burden (Beta = 0.01, P = 0.045).*

- **Ages in association between germline variant and somatic mutational burden:** One more thing we'd like to add is that this direction of correlation is affected by ages. As depicted in Figure 2d. a notable positive correlation exists between the germline variant burden and the somatic mutational burden, particularly in early-onset adult cancer (those below 40 years of age). For example, patients in their twenties exhibit a higher correlation coefficient between germline and somatic mutations compared to those in their forties. This is a remarkable finding since there is generally a positive association between age and the somatic mutational burden, while a negative or neutral correlation with germline variants is expected (i.e., as age increases, the somatic mutational burden tends to increase, but the germline variant burden does not). In essence, while age influences these two variables in contrasting manners, there remains a positive correlation between them in patients with younger ages. Collectively, these insights underscore the pivotal role of germline variants in influencing somatic mutations among younger populations, including both pediatric patients and young adults.

- **Distinctive from Qing et al., our study bears several notable differences:**

1. Our variant filtering process differed somewhat. Qing utilized the MetaSVM score, while we employed

a more recent and high-performance in silico tool, the REVEL[2] database when counting pathogenic missense variants. Additionally, to further select functional germline variants, we attempted to minimize false variants by ensuring that they were present in more than 10% of each cohort. As a result, while Qing's study reported a range of 79−239 variants, our count did not exceed 150 in the TCGA.

2. Secondly, unlike Qing, we did not exclusively analyze cancer genes. This decision was based on our intent to compare the results from pediatric cancer, neuroblastoma, with those from adult cancers. We anticipated that the genes and related pathways involved in these two types of cancer would significantly differ. By adopting this approach, we aimed to reduce the error of arbitrarily selecting genes. However, it's crucial to recognize, as Qing highlighted, that not every gene holds equal weight in cancer research. Given our expansive analysis that incorporated all the genes, it's conceivable that the correlations we observed were less pronounced. While focusing on specific genes, as Qing did, might have yielded more pronounced correlations, we believe that selecting the appropriate genes for a meaningful comparison between the diverse 31 types of cancers in TCGA and pediatric neuroblastoma demands rigorous validation.

We hope this translation captures the essence of our research distinction from Qing's. Taking the reviewer's suggestion into account, we further elucidated this point in the discussion section as below.

Page 8, lines 229−237: "*Qing et al*[3]*. have clearly described the association between germline variants and somatic mutations in adult-onset solid cancer, whereas our variant filtering process differed from theirs. In our analysis, we opted for the REVEL*[2] *method, which has demonstrated superior performance in comparison to MetaSVM*[4]*, employed by Qing et al. Additionally, we further refined our selection by excluding variants that were present in more than 10% of each cohort, aiming to minimize false positives. Consequently, our findings present a narrower range of variants, with no more than 203 variants per patient in the TCGA cohort, in contrast to the 79−239 variant range reported in Qing's study. Another difference is, our study's focus on pediatric patients and the inclusion of a wide array of genomic data, not limited to cancer-specific genes. This likely accounts for the observed weaker correlation between germline variants and somatic mutations compared to the associations reported by Qing et al.*"

Comments 1-5: CPG analysis was based on pFGVs, which is a non-standard approach as the community primarily focuses on using P/LP variants for enrichment analysis. **Was the clinical outcome and enrichment compared to the healthy population driven by P/LP variants in CPG?** A comparison is needed to clarify the role of pFGVs versus P/LP variants.

Response 1-5: We appreciate the reviewer's query regarding the role of pFGVs in our analysis. It is imperative to clarify that our approach was not to challenge the established significance of P/LP variants in genetic studies, but rather to complement it by also considering pFGVs, which may provide additional insights, especially in complex pediatric cancer settings. In the results section of our manuscript, we already thoroughly examined the impact of P/LP variants on clinical outcomes. We identified a noteworthy association with OS in our patient cohort, affirming the established understanding of the clinical relevance of these variants. Conversely, this association

was not as pronounced when analyzing PFS. In contrast, when we broadened our analysis to include pFGVs, we observed an enhanced correlation with PFS. This suggests that pFGVs capture a subset of clinically relevant genetic variations that P/LP variants alone may overlook.

In response to the reviewer's comment, we have now included in the revised manuscript the observed higher prevalence of P/LP variants in our neuroblastoma cohort, as anticipated by the reviewer. While this enrichment underlines the importance of P/LP variants, the presence of such variants in cancer predisposition genes (CPGs) was limited to approximately 10% of patients, hinting at the presence of a broader spectrum of functional genetic variations that may play a role in neuroblastoma.

Page 6, lines 164−167: "*However, it was important to note that when refining our analysis based on the American College Medical Genetics (ACMG) guidelines for clinical interpretation[5], which focuses sole on pathogenic or likely pathogenic (P/LP) variants in CPGs, there was a pronounced enrichment of these variants in neuroblastoma within the SMC cohort compared to the general population (P = 0.016; Odds Ratio, 2.13; 95% CI, 1.10−3.91).*
"

Page 6−7, lines 177−181: "*When we considered only P/LP variants according to the ACMG guidelines, a more pronounced distinction was observed in the family history of cancer between patients harboring P/LP variants in CPGs and those without such variants (91% vs. 44%, P = 0.023). However, the survival differences in survival outcomes were significantly only for only OS (log-rank P = 0.009; Extended Data Fig. 5a), and not for PFS (log-rank P = 0.308; Extended Data Fig. 5b).*"

Lastly, we have added statements in the discussion section that pFGVs cannot replace P/LP variants according to the ACMG.

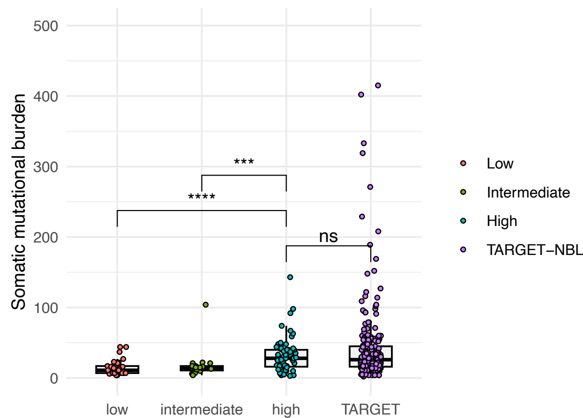
Page 9, lines 261−263: "*Finally, our pFGVs cannot supplant or diminish the importance of P/LP variants as defined by the ACMG. This is because a family history of cancer, enrichment and some observed survival differences are more pronounced when adhering strictly to P/LP classifications compared to pFGVs*."

Minor points:

Comments 1-6: A comparison of somatic mutation burden in SMC with other published neuroblastoma data set is needed to ensure the accuracy of the data analysis.

Response 1-6: While we have not made alterations to the manuscript following your query, we offer the following detailed explanation to elucidate our findings. We undertook a comparison of the somatic mutational burden in our study's neuroblastoma cohort with that of the TARGET dataset. The analysis, depicted in the below figure, shows that patients with high-risk neuroblastoma in the SMC dataset exhibit a higher mutation relative to those in the low or intermediate risk categories. However, the comparison with the TARGET dataset, which is composed exclusively of high-risk cases, did not reveal a significant difference. Corroborating the literature, Pugh et al[6]. found a median of 18 exomic mutations per neuroblastoma tumor in 240 patients. Our findings are closely aligned, with the SMC dataset showing a median of 17 mutations per tumor and the TARGET dataset showing 26 mutations per tumor across 220 patients. We hope that this comparison has provided the reviewer with confidence in our

analysis.



*** $P \leq 0.001$

Comments 1-7: Figure 2d. TARGET data set only includes the TARGET neuroblastoma based on the description in Methods. Please clarify in the label and figure legend.

Response 1-7: Thank you for your suggestion. Based on your feedback, we have made the necessary corrections.

Comments 1-8: Abstract—all descriptive without the statics to justify the importance.

Response 1-8: In accordance with the reviewer's suggestions, we have tried to modify abstract. However, However, the Nature Communications submission guidelines limit the abstract to about 150 words and require that it provide general information and a brief non-technical summary of the results. Therefore, the abstract could not be modified to address the reviewer's comments.

Comments 1-9: Need to clarify the data source for CPG. The results shown in lines 122-123 indicated that 109 CPGs were analyzed when reference to Supplementary Table S1 which listed all 733 Cancer Gene Census from COSMIC and the authors need to show only the CPG genes in this table (presumably those labeled as Germline in second column).

Response 1-9: Our apologies for any confusion regarding the CPG gene list. We have amended the Supplementary Table 1 to ensure the distinction between CPGs and the rest gene set is clear. We have kept rest gene list because it was used in the analysis (Extended Data 5a, b). Thank you for bringing this to our attention.

Comment 1-10: Figure 1a. Population filtering. KRGDB 1100 <1% was used for filtering SMC cohort but not the TARGET/TCGA cohort. This can cause potential problem with the rare variants in Korean/Asian patients involved in TARGET/TCGA as one variant may be considered as pFGVs in one cohort but non-pFGV in the other. Please evaluate this scenario.

Response 1-10: The reviewer raised a valid point concerning the use of KRGDB 1100 <1% for filtering the SMC cohort but not the TARGET/TCGA cohort. Indeed, there is a possibility that the TARGET/TCGA data might not have been as thoroughly filtered for variants as our SMC cohort. As such, direct comparisons between cohorts pose significant limitations in our study.

Within the TARGET cohort, the challenges of accurately defining individual specific ethnicities meant we couldn't employ further filtering parameters. However, as only three individuals in the TARGET cohort were identified as Asian, the potential discrepancies in variant identification, as pointed out by the reviewer, might be relatively minor. Our primary goal was centered on identifying intra-cohort trends, which we think alleviates some of the concerns raised. Nevertheless, we agree with your observation, and we acknowledge that this represents another potential limitation of our study. We will make sure to address this point in our manuscript. Thank you for bringing it to our attention.

Page 9, 256–259: "*Additionally, the total count of germline variants identified could have been influenced by the specific experimental design and the variant filtering processes applied, which varied across cohorts. Therefore, the germline variant burden must be interpreted with caution at an individual level. Such variance in methodology also renders direct comparisons between cohorts challenging*."

Comments 1-11: Supplementary Table S1: Gene symbols for SEPT5, SEPT6 and SPET9 are in date format in excel sheet.

Response 1-11: Thank you for pointing out the oversight. We have corrected the gene symbols for *SEPTIN5*, *SEPTIN6*, and *SPETIN9* that were mistakenly formatted as dates in the Excel sheet. We appreciate your meticulous review.

<u>Reviewer #2</u> (Remarks to the Author): Expert in neuroblastoma genomics and clinical research

General comment:

Review of Germline Functional Variants Contribute to Somatic Mutation and Outcomes in Neuroblastoma

Seo et al performed germline whole exome sequencing of a cohort of 125 patients with neuroblastoma from both HR and IR/LR risk groups and showed that the burden of putative functional germline variants (predicted LOF and missense mutations - termed pFGVs by Seo et al) is associated with the somatic mutation burden in neuroblastoma as well as having a prognostic impact in neuroblastoma.

They then go on to demonstrate that pFGVs in cancer predisposing genes (CPGs) are enriched in neuroblastoma (both using their own cohort as well as TARGET-NBL; comparing with KOREA1K and TCGA, respectively) and then demonstrate that pFGVs (specially when additional filtering according to the ACMG clinical criteria for likely pathogenic and pathogenic (LP/P)) alter prognosis in neuroblastoma — predominantly in MYCN-negative cases.

The paper is in general well structured and easy to read, and in my mind especially that last results where Seo et al demonstrate that presence of LP/P-variants in CPGs affects overall survival are quite interesting, also in a clinical context.

I do, however have some questions regarding methodology that I feel needs to be addressed:

Response: We thank the reviewer for the positive evaluation of our manuscript. We are pleased that our research on the role of germline functional variants in neuroblastoma has resonated with the reviewer. We have addressed the comments raised with due diligence and hope that our responses will provide further clarification for the reviewer.

Comments 2-1: In their testing of the burden of germline variants and its association with clinical variables they dichotomise the germline variant burden — this is not statistically sound practice, they should test the germline variant burden as a continuous variable here.

Response 2-1: We are grateful for the reviewer's constructive critique. However, it is important to note that in our comparative analysis of clinical variables (excluding outcomes) with germline variant burden, we indeed treated the germline variant burden as a continuous variable. This has been further elucidated in our revised analyses and additional investigations, which can be found in Extended Data Figure 3. Regarding the dichotomization of germline variant burden in the context of clinical outcomes, we have provided a detailed explanation and addressed the statistical considerations in **Response 2-8.**

Comments 2-2: In general, one needs to be wary of potential differences in ethnicity when doing genetic comparisons between cohorts, have the authors performed any analysis to this end? (e.g. PCA-plots of common SNPs) if not, this needs to be done and included in the paper.

Response 2-2: We sincerely appreciate the reviewer for highlighting this crucial aspect of our study. As recommended by the reviewer, we have added PCA plots using SNPs and plots showing estimated ethnicity for both the SMC and TARGET cohorts. We utilized the EthSEQ package (v 3.0.2) in R for this analysis, using genotype data for 10,000 exonic SNPs provided in EthSEQ as a reference model. Detailed methods have been added in the method section. As expected, the SMC cohort consists solely of East Asians. In contrast, the TARGET NBL dataset comprises individuals of diverse ethnicities, and there were discrepancies between the reported ethnicity and ethnicity estimated from SNPs. The findings from this analysis have been incorporated into Extended Data Figure 8. Lastly, TARGET data has been reanalyzed using ethnic-matched filtering process.

Comments 2-3: The methods section needs to be expanded with more detail. What exome enrichment kit was used? They also state that some of the tumor samples (from where somatic mutations were called) was from FFPE tissue, it would be very vary of comparing (somatic) mutational burdens between fresh frozen samples and FFPE samples — it needs to be acknowledged which SMC samples had tumor DNA extracted from FFPE tissue samples.

Response 2-3:

- **Exome enrichment kit**: In response to the inquiry regarding the exome enrichment kit used: In our experiment, tumor and matched normal DNA were enriched for exon regions using the SureSelect XT Human All Exon V5 kit, both from Agilent Technologies Inc., Santa Clara, CA, USA.
- **Pertaining to the source of tumor samples:** We acknowledge and clarify that we had a total of 33 FFPE samples and 92 samples from fresh tissue.
- **Concerning the comparison of somatic mutational burden between FFPE and fresh tissue samples**: We carried out a comparative analysis, and our results did not show a significant difference in the somatic mutational burden between the two types of samples. Specifically, the T-test results were as follows: FFPE samples had a mean somatic mutational burden of 27.55, while fresh tissue samples had a mean of 22.37. The resulting P-value was 0.264, indicating no significant difference.

We hope this clarifies the methods employed in our study and addresses your concerns. We appreciate the opportunity to provide further detail. We have now added these information in the Method section as follows:

Page 10, 284–287: "*Genomic sequencing was performed on tumor and normal DNA extracted from fresh frozen (74%) or formalin-fixed paraffin-embedded tissues (26%) and mononuclear cells from peripheral blood, respectively, using a QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA). Tumor and matched DNA were enriched for exon regions using SureSelectXT Human All Exon V5 kit (Agilent Technologies Inc., Santa Clara, CA, USA).*"

Comments 2-4: To what depth was the germline and tumor samples from the SMC cohort sequenced?

Response 2-4: For the SMC cohort, the median coverage depth of exome sequencing for the normal (germline) samples was 74X. In contrast, the tumor samples exhibited a median coverage depth of 114X.

Comments 2-5: Re-calling the TARGET-NBL data was in my mind absolutely the right thing to do, and they also correctly note (and mitigate) the known issues with the somatic mutations in the TARGET-NBL dataset. Furthermore, how does the variant calling of the TCGA samples differ from the TARGET-samples? Ideally they should be processed through the sample variant calling pipeline. At the very least, the differences needs to be clearly stated.

Response 2-5: Thank you for highlighting the importance of detailing the differences in variant calling between the TCGA and TARGET samples. For the TCGA samples, we relied on the VCFs from the MC3 dataset. As detailed in the paper "Scalable Open Science Approach for Mutation Calling of Tumor Exomes Using Multiple Genomic Pipelines" by Kyle Ellrott et al[7]., the TCGA MC3 dataset underwent variant calling with a range of tools, including Mutect, MuSe, Radia, Sniper, and Varscan. Additionally, the dataset was refined by addressing and eliminating OxoG errors using various tools, one of which is the DetOxoG. Conversely, for the TARGET samples, the absence of a refined VCF comparable to the TCGA MC3 dataset led us to access the raw FASTQ files directly. We then performed variant calling as delineated in our methods section, using a strategy derived from literature recommendations. To enhance clarity and transparency in our paper, we have now added these information in the method section.

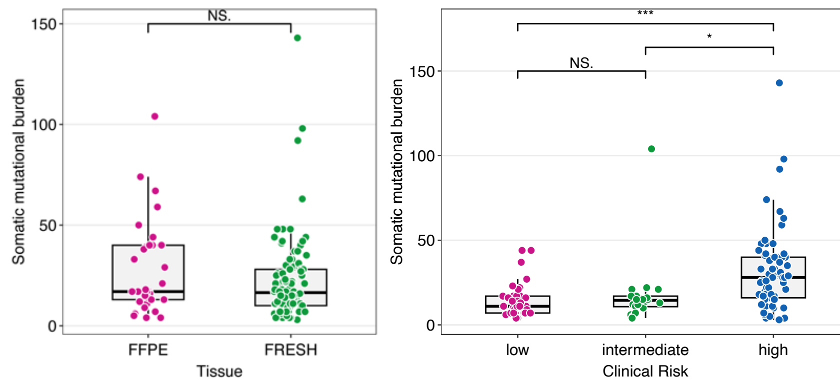
Page 12, 344–346: "*Somatic mutations in the TCGA were obtained from TCGA PanCancer Atlas MC3 set[8], which is the result of applying an ensemble of seven mutation-calling algorithms, complete with scoring and artifact filtering[7]. Then we applied the sample somatic mutation call pipeline used in the SMC cohort.*"

Comments 2-6: The code used needs to be publicly available on GitHub or FigShare.

Response 2-6: In accordance with the request, we have now uploaded all the essential codes required for the analysis and figure generation to our GitHub repository (https://github.com/SGIlabes/NBL_Germline).

Comments 2-7: In Figure 1C: there are some clear outliers in somatic mutational burden, are these variant calls derived from FFPE tissues? Did the patients have germline mutations in CPGs that are known to give an increased number of somatic mutations?

Response 2-7: Thank you for your insightful observation regarding the outliers in somatic mutational burden shown in Figure 1C. You are correct in noting the presence of outliers; however, these do not originate from FFPE tissues. It's worth highlighting that the somatic mutation burden appears more influenced by factors such as the patient's stage and *MYCN* amplification, which are components of the clinical risk group. The correlation between these clinical risk factors and the somatic mutation burden is elucidated further in the figure below.



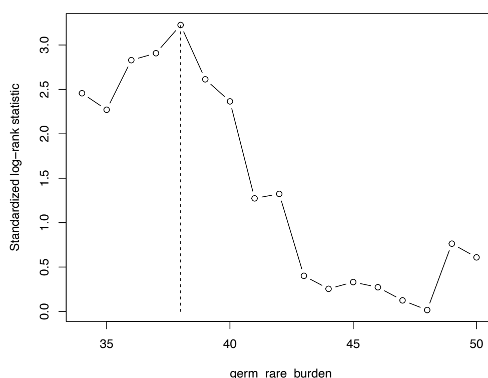
* $P \leq 0.05$, *** $P \leq 0.001$

Comments 2-8: Figure 3: Need to treat the Germline variant burden as a continuous variable and not dichotomise in to high and low (causes loss of information)

Response 2-8: We appreciate the reviewer's critical viewpoint on our methodological approach. While we acknowledge the statistical impact of treating germline variant burden as a continuous variable on survival, we opted for dichotomization in this instance due to the heterogeneous nature of variant impacts. This variability arises from differences in gene function and mutation severity, which are not uniform across the variants. Our analytical model was thus designed to identify broad, overarching trends rather than the nuanced impact of each variant. We believe this approach still yields meaningful insights, although we recognize the potential for further refinement. Identifying genes with a strong influence on patient prognosis and excluding those with minor effects could enhance our model, potentially allowing for continuous variable analysis to yield meaningful results in the future.

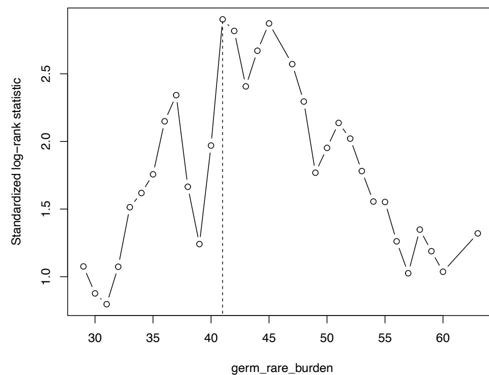
To address the reviewer's point, we have supplemented our analysis with additional figures generated using maximally selected log-rank statistics. These figures elucidate that beyond the average or median, there are multiple threshold points that could demarcate significant survival differences between patients with high and low germline variant burdens. We hope this addresses the concerns raised.

<SMC>



SMC average/median: 41

<TARGET>



TARGET average: 45, median 41

Nevertheless, to address the reviewer's comments, we also have conducted a Cox Proportional-Hazards analysis considering the germline variant burden as a continuous variable. As indicated in the table, when we initially analyzed the germline variant burden as a continuous variable, we encountered challenges in discerning clear survival differences. This underscores the complexity of the individual variant effects.

	Hazard ratio	*P* value
SMC (PFS)	1.05	0.111
TARGET (OS)	1.00	0.256

Comments 2-9: Figure 4: some cases with germline mutations in genes known to cause an increased mutational load - did they perform further analysis with this subgroup removed?

Response 2-9: Thank you for your inquiry. We have already addressed this concern in the main manuscript. Specifically:

1. Page 4, 105−106: This correlation maintained nominal statistical significance in patients without pFGVs in DNA damage repair (DDR) genes (Pearson's r = 0.23; P = 0.032; Extended Data Fig. 1a).
2. Page 4, 115−118: This correlation persisted in patients without pFGVs in DDR genes (Spearman's ρ = 0.33; $P < 0.001$; Extended Data Fig. 2a), as well as in analyses that excluded outliers identified using a Z-score threshold of 3 (Spearman's ρ = 0.24; $P < 0.001$; Extended Data Fig. 2b).

Comments 2-11: Figure 5D: also dichotomisation — needs to be re-analyzed using the Germline Variant burden as a continuous measure.

Response 2-11: We've elaborated on this in detail in our responses to Comments 2-8 and hope this provides clarity regarding our approach in Figure 5D as well.

Comments 2-12: Figure 6: this is really interesting and to me the key finding!

Response 2-12: We genuinely appreciate your feedback. We are hopeful that these findings will inspire further exploration through extensive prospective studies across diverse pediatric cancers.

Comments 2-13: Extended Data Fig.1: Why are they mixing Pearsons r with Spearman's rank correlation coefficient? (And why log10-transform data when using a non-parametric correlation measure such as Spearman's?)

Response 2-13: Thank you for pointing out the apparent discrepancy. We opted for Spearman's rank correlation coefficient for the TARGET and TCGA datasets specifically because the distributions of both somatic and germline variant data in these datasets did not adhere to the assumptions of normality, a prerequisite for Pearson's correlation. On the other hand, the log10-transformation was implemented not as a prerequisite for the Spearman's correlation but to ensure visual consistency across the figures when presenting the SMC data. Our intention was to provide clarity and uniformity in presentation while simultaneously selecting the most appropriate statistical test for the nature of the data at hand. We trust this clarifies our approach and rationale.

Comments 2-14: Extended Data Fig.5: This is really interesting and could be very useful in a clinical setting in a short timeframe. The authors should perform the same analysis also on the TARGET-NBL cohort to se if it holds.

Response 2-14: Thank you for your insightful comments. Based on your recommendation, we performed survival analysis in the TARGET-NBL cohort following the ACMG guidelines. About 17% of patients exhibited PV or LPV in our CPG gene list. Consistent with our observations in the SMC data, the survival outcome, particularly in OS, was significantly influenced by the presence of PV/LPV.
To reflect these findings, we incorporated the following sentence into our manuscript:

Page 7, 193−193: "*When assessing pathogenicity as per the ACMG guidelines, the TARGET cohort displayed significant differences in OS but not PFS as SMC cohort (OS, log-rank P = 0.025; PFS, log-rank P = 0.817).*"
We appreciate your valuable feedback and hope that these additional analyses strengthen our manuscript's overall contribution to the field.

Reviewer #3: Expert in neuroblastoma genetics, predisposition, and therapy

General comments: The authors performed whole-exome sequencing of 125 patients with neuroblastoma from South Korea to study the role of putatively functional germline variants (pFGVs) in neuroblastoma pathogenesis. This study focused on 109 cancer predisposition genes (CPGs) listed in the Cancer Gene Census (CGC) from the Catalogue Of Somatic Mutations In Cancer (COSMIC) database (page 5). The CGC lists 738 genes, so presumably the 109 genes included in this study were chosen because they were CPGs, many of which (80) are possibly involved in DNA stability and repair mechanisms. They found a direct correlation between pFGVs and somatic mutations in tumors, as well as with patient outcome (higher pFGVs correlated with worse outcome). Similar results were seen in a separate neuroblastoma cohort, but not seen when analyzing an adult cancer cohort. They conclude that the combination of germline and clinical risk factors improves survival predictions.

Response: We appreciate the thorough review. The reviewer is correct that we selected 109 CPG genes from the CGCs. However, we also would like to add that **before focusing on CPGs, we initially counted germline variants at the whole-exome level.** Subsequently, our analysis also encompassed the significance of CPGs. Additionally, we have prepared responses by breaking down the reviewer's profound concerns into parts according to context, for a detailed reply. We seek your understanding in advance.

Comments 3-1: The connection between the burden of pFGVs and the development of neuroblastoma in this study is conjecture at best and hardly actionable, as there are a number of different CPGs affected, and none have a direct association with neuroblastoma predisposition. The burden appears to be a single germline change in most cases (34 of 39 according to Figure 4), two mutations in 5 other cases, and most are missense mutations. It is difficult to understand **how a mutation in any one of the genes listed** selectively increase **the risk of neuroblastoma.** The pFGV burden is not explored in much detail, and according to Figure 4 seems to involve only a single gene in most cases. Moreover, it is not clear if they are proposing the use of pFGV burden only as a prognostic marker or also as an insight into cancer predisposition. The top genes involved include FAT1, MLH2, MSH2, BRCA2, MAX, and TP53, most of which are known to be associated with DNA instability or repair, so a germline mutation, especially a truncating mutation, could be a contributing factor to increased somatic mutations in tumors. In addition, the use of pFGV "burden" as a prognostic marker is hard to understand or study, when there are dozens of serum biomarkers, tumor expression profiles, or other predictive markers and algorithms that have shown similar or stronger predictors of outcome.

Germline mutations in a few genes predispose to development of neuroblastoma, such as ALK and PHOX2B, with high penetrance (~50% each). There are a few other genes associated with syndromes that also predispose to neuroblastoma, such as CDKN1C mutations in Beckwith-Wiedemann syndrome and KRAS in Costello syndrome, in which the penetrance of neuroblastoma is lower (1-5%). Finally, there have been dozens of genes identified by GWAS studies that were called neuroblastoma "susceptibility" or "predisposition" genes, but most genes implicated by GWAS have been one-off observations with weak effects, and some of the SNPs are near but not even in the gene, so they are not really actionable as CPGs without further investigation.

Response 3-1: We appreciate the reviewer's insightful comments. We apologize for any misunderstanding regarding the calculation of the pFGV burden**. It is crucial to clarify that the pFGV burden was not determined exclusively within CPGs, but rather across over 19,000 genes for each patient, aiming for a comprehensive assessment of germline variants**. Moreover, protein-truncating variants rather than missense variants are frequent in most patients (Fig. 1b). This approach was taken as most neuroblastoma patients usually do not possess pathogenic germline variants in CPGs. Our hypothesis posited that **even variants not directly associated with neuroblastoma risk could influence the characteristics and outcomes of neuroblastoma**, without necessarily increasing the risk of developing the disease. In other words, we do not assert that each variant in these genes individually increases the risk of neuroblastoma. Instead, we sought to shed light on the potential role of germline variants in individuals who have already developed neuroblastoma. In this context, we discovered evidence suggesting a correlation between the germline variant burden and both somatic mutations and clinical outcomes. Subsequently, **we narrowed our focus to CPGs to hone in on genes with the most plausible potential roles, as depicted in Figure 4**. Approximately one-third of patients have pFGVs in CPGs, and as the reviewer rightly observed, most of these CPGs are related to DNA repair mechanisms. It is also notable that individual patients typically do not share the same variants or even the same affected CPGs. Lastly, it's important to differentiate that our pFGVs are exonic variants, potentially more impactful due to their likelihood of affecting protein-coding regions. This contrasts with many GWAS-identified SNPs.

Comments 3-2: There are a large number of clinical, laboratory, genetic, genomic, expression, radiographic, pathologic, and other predictive markers of neuroblastoma prognosis. Furthermore, there have been somatic genetic studies identifying single genes (e.g., MYCN, ALK) that have prognostic value, but there have also been dozens or hundreds of reports of expression studies of single genes, small groups, and larger panels that predict outcome in neuroblastoma, but **essentially none of these have stood the test of time or become implemented in national or international cooperative group studies.** Indeed, **what is really needed for neuroblastoma as well as other pediatric tumors is more effective, less toxic therapy, not more prognostic markers or predictive algorithms.**

Response 3-2: We wholeheartedly agree with the reviewer's perspective, particularly from a clinician's standpoint, and recognize that there is a significant need for novel treatments[9], rather than more biomarkers, especially for pediatric patients. We acknowledge the existence of numerous established predictive markers and algorithms for neuroblastoma prognosis, such as the well-recognized INRG risk classification system[10,11], which considers a variety of strong risk factors. However, it is important to highlight that even with the most intensive treatments, the relapse rate is nearly over 50%, particularly in high-risk neuroblastoma cases. This underscores the need for identifying additional risk factors to predict neuroblastoma outcomes more accurately.[9] Given that most germline genetic studies have centered around the risk of developing the disease, we have shifted our focus to clinical outcomes. Our research aimed to explore factors beyond the established clinical and somatic determinants to gain deeper insights into neuroblastoma prognosis. Through this, we hope to contribute to a more comprehensive understanding of the complex interplay of genetic factors in neuroblastoma and offer a fresh perspective that may complement and enrich the existing body of knowledge on the disease.

Comments 3-2: The patient cohort on which they focused was 125 neuroblastoma patients, but they do not specify over what period of time these patients were diagnosed, how they were selected, or if they were representative. Given that about half were high-risk and half were low or intermediate risk, they are presumably representative, but it would be helpful to clarify this.

Response 3-2: Our study focused on a cohort of 125 neuroblastoma patients, whose diagnoses spanned from 2015 to 2021. We carried out WES on all available neuroblastoma tissue samples during this period. Initially, 145 peripheral neuroblastic tumors were identified for WES. From these, we excluded 6 ganglioneuroma cases and 9 cases where the tumor tissue was obtained after relapse or progression. Furthermore, one patient was excluded due to having unmatched pairs of blood DNA and tumor DNA, as confirmed by NGSCheckMate[12]. We also made a conscious decision to include only tumors obtained from the primary site, leading to the exclusion of an additional 4 patients. Consequently, our analysis focused on the remaining 125 cases. To enhance clarity and transparency in our paper, we have now added this in the method section.

Page 10, 276−279: *"We analyzed blood and tissue DNA from 125 neuroblastoma patients diagnosed between 2015 and 2021, initially identifying 145 patients with peripheral neuroblastoma tumors. After excluding ganglioneuroma cases (n=6), tumors obtained post-relapse (n=9), patients with unmatched DNA pairs confirmed by NGSCheckMate534 (n=1), and non-primary site tumors (n=4), our analysis focused on the remaining 125 cases."*

Comments 3-3: Also, they mention the "clinical" risk factors of age, stage, and MYCN status (**page 6**), but current risk prediction algorithms in the US, Canada, Europe, and Japan use more complex algorithms. Figure 4 does list age, sex, stage, risk group, path, and MYCN status, so presumably they used all of these as their "clinical" risk markers, **so this should be clarified in the text**. Details of the patient characteristics are shown in extended table 1, but they should indicate whether the breakdown of different markers are similar to an unselected series of patients.

Response 3-3: Firstly, we have provided data on the relationship between various clinical variables and our analyzed germline variant burden as well as pFGVs in CPGs, as presented in Extended Data Table 2 and Extended Data Fig. 3. While we are not entirely certain that we have fully grasped the nature of the question, we will proceed under the assumption that it pertains to our prediction mode (Fig. 7). To address the reviewer's concerns regarding the use of only age, *MYCN* status, and stage as clinical factors in our model, we have prepared a detailed response.

Our study initially focused on these three factors based on their well-established significance in neuroblastoma risk stratification. Despite identification of new biomarkers, age, stage, and *MYCN* status remain the most highly prognostic and are the foundation of current neuroblastoma risk stratification.[13,14] These factors are also predominantly used in major clinical studies such as GPOH NB97[15], NB2004[16], SIOPEN HR-NBL1[17], and COG A3973[18], and ANBL0532.[19] In addition to what has been mentioned, it is important to note that our model is based on patients from our institution, and in practice, we categorize and treat patients using these three factors. The outcomes of this approach have been reported in our NB-2004[20], 2009[21], and 2014 (unpublished) studies.

Although the INRG classification of 2009[22] introduces additional factors like 11q aberration, ploidy, and tumor differentiation, it is evident that these factors do not replace age, *MYCN* status and stage, particularly in determining high-risk categories. With the 2021 update to neuroblastoma risk stratification[23], new criteria such as tumor size and INPC-based reclassification of some cases from intermediate to high risk have been included. However, this does not diminish the relevance of the traditional factors we employed. Considering potential collinearity among various variables (such as *MYCN* amplification and pathology), we chose a model that emphasizes the most influential and established factors to ensure robustness and clarity in our predictive analysis. In the future, if prospective studies are conducted in accordance with the newly updated risk stratification, in conjunction with germline genetic research, we may be able to determine the impact of germline genetics on the most up-to-date risk stratification.

Comments 3-4: My other concerns about this manuscript are that the effect sizes and p-values are almost all very weak. Many of the analyses presented had marginally significant p-values, such as 0.018, 0.032, 0.024, etc., and none of these would survive a Bonferroni correction. They did apply this correction to the added predictive value of germline risk factors in neuroblastoma (page 6 and Figure 7), but not to other statistical analyses.

Response 3-4: We greatly appreciate the reviewer's input and acknowledge the concerns raised regarding the effect sizes and p-values in our study. It is indeed the case that the distribution of germline variants in our study is relatively narrow, which has led to smaller effect sizes in terms of correlation. The modest p-values observed in our analyses are a reflection of this. However, we made concerted efforts to mitigate biases, such as by performing subgroup analyses excluding patients with defective DDR genes, and by adjusting for potential confounders in both correlation and survival analyses. Moreover, the valuable feedback from the reviewers has led us to refine our analysis, resulting in more significant outcomes.

Regarding the issue of multiple testing, our primary analysis was focused and did not involve extensive testing across numerous divergent hypotheses. The main results, presented in the core figures of our study, incorporate analyses of all patients across the three datasets. However, it is to be noted that in Fig.6, Extended Data Fig. 1, 2 and 5, which include subgroup analyses, multiple testing did occur. We recognize, particularly in the case of Fig.1a, that the results would not maintain statistical significance under Bonferroni correction. We have now made this acknowledgment explicit in the manuscript.

Page 4, 105−106: "*This correlation maintained nominal statistical significance in patients without pFGVs in DNA damage repair (DDR) genes (Pearson's r = 0.23; P = 0.032; Extended Data Fig. 1a).*"

Page 7, 192-193: *"These results were also consistent in the SMC cohort with nominal statistical significance (Fig. 6c, 6d).*"

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have addressed all the questions raised in the previous review except for a minor comment related to the abstract "Abstract—all descriptive without the statics to justify the importance". The authors opted not to revise to keep within the 150-word limit. I would suggest that statements in abstract listed below leaves the impression of lack of scientific rigor of the study and the authors should consider revising. Some part of the abstract (e.g. you do not need to spell out the full TARGET in the abstract to save words) can be made more succinct so that odds ratio, p values can be presented to quantify the significance of their findings.
"The enrichment of pFGVs in cancer predisposition genes was evident in neuroblastoma compared to that in healthy and adult-onset cancer populations, and their presence had prognostic significance in neuroblastoma. The combination of germline and clinical risk factors improves survival predictions."

Reviewer #3 (Remarks to the Author):

I think the authors have done a very thorough and detailed analysis of germline variants in cancer predisposition genes in patients with neuroblastoma. They infer that these germline functional variants may contribute both to somatic mutations in tumors and to patient outcomes. The authors have addressed all the comments and concerns, and I have no additional comments.

Reviewer #4 (Remarks to the Author): Expert in neuroblastoma genomics; replaces Reviewer #2

Seo and colleagues perform germline whole exome sequencing in a cohort of 125 neuroblastoma patients from Korea, termed the SMC cohort. Importantly, this adds an Asian cohort to the two large neuroblastoma germline studies already published to date (Bonfiglio et al, eBioMedicine 2022: n=664 Italian patients and Kim et al, JNCI 2023: n=786 patients from North America; this is an extended version of the TARGET project). The authors identify putative functional germline variants (pFGVs; predicted LOF and missense variants present in < 1% of ExAC) and test for association with tumor mutation burden, clinical variables, and outcomes as well as enrichment compared to controls and adult cancers. They report a positive correlation between germline pFGV burden and tumor mutation burden in their cohort. This is replicated using exome sequencing data (n=220) from the TARGET project. No association was observed between pFGVs and clinical variables in the SMC cohort, consistent with prior reports by Bonfiglio and Kim. A statistically significant association was observed between pFGV burden and progression free survival (PFS) in the SMC cohort and overall survival (OS) in the TARGET data when pFGV burden was dichotomized using the mean burden as a cutoff; however, this did not remain significant in an analysis where pFGV burden was evaluated as a continuous variable (Response 2.8).

Finally, the authors perform a focused study of 109 cancer predisposition genes (CPGs). There was a trend toward enrichment of pFGVs in the SMC cohort compared to KOREA1K controls; however, this only became statistically significant when further restricted to pathogenic or likely pathogenic (P/LP) variants in CPGs according to ACMG criteria. The TARGET cohort was not compared to a "healthy" control cohort, but rather was compared to adult cancers in TCGA. This analysis showed an enrichment of pFGVs in TARGET vs. TCGA. However, it is noted that neither the control cohort (KOREA1K) nor the adult cancer cohort (TCGA) were processed in the identical manner as the neuroblastoma patient data. Finally, the authors report results of various analyses investigating the role of pFGVs in patient outcomes, including survival. They conclude the pFGVs are prognostic in neuroblastoma, particularly in the MYCN non-amplified subset.

While pFGVs have been studied in other cancers, most studies in neuroblastoma have focused on P/LP variants in CPGs. An exception to this is Bonfiglio et al, eBioMedicine 2022, where whole exome sequencing was performed on 664 neuroblastoma cases and 822 controls followed by analysis of a broad set of rare variants annotated as pathogenic according to M-CAP and CADD prediction (not referenced in manuscript). This study demonstrated enrichment of these variants compared to controls. Otherwise, this is the first broad analysis of pFGVs in neuroblastoma. The finding that pFGV burden is associated with tumor mutation burden is novel. The results reported regarding enrichment of pFGVs are less compelling and should be expanded on to use controls and adult cancer data fully processed using the same pipelines. Utilizing publicly available variant calls can result in spurious results (Kim et al PLOS One, 2023). The finding that pFGV burden associates with worse outcome is also of interest. However, several portions of the results presented do not control for the influence of known P/LP variants in CPGs, which have been demonstrated to associate with worse outcomes in a study of 786 neuroblastoma cases, including the TARGET cohort (Kim et al, JNCI 2023; referenced as Kim et al, MedRxiv 2023 in the initial submission of this manuscript). The analyses also rely on dichotomized data rather than considering pFGV as a continuous variable. Taken together, this raises questions regarding the true contribution of pFGVs in neuroblastoma beyond P/LP variants in CPGs. Overall, the manuscript is well written and presents some interesting results. However, the enrichment and survival portions of the manuscript require further clarification to discern the true contribution of pGFVs. In addition, findings in the paper are not set in accurate context leading to overstatement of novelty of results. This should be addressed and clarified throughout the paper.

MAJOR:

1.Figure 2 – the somatic mutation burden for TARGET exomes presented in panels A and B do not appear to agree. based on a log 10 transformation. The base of the logarithm in Panel B may be mislabeled, or there is a larger issue that needs to be addressed.

2.Figure 2D – why does this only include TARGET exomes? What about the SMC exomes? Are results consistent?

3.The results section labeled "pFGVs in CPGs are enriched in neuroblastoma" is misleading. The abstract and results state there was an enrichment of pFGVs in CPGs in SMC cohort compared to KOREA1K.

However, the p-value is not statistically significant. This should be reworded, including the result heading and abstract. In fact, it was only when restricting to P/LP variants in CPGs according to ACMG guidelines that a significant enrichment was observed. This finding has been reported in a cohort of 786 neuroblastoma patients, including the TARGET exomes (Kim et al, JNCI – referenced in the original manuscript as Kim et al, MedRxiv 2023). While it is important that the finding of CPG P/LP variant enrichment was replicated here in the SMC cohort, it seems plausible that the trend toward significance for the broader set of pFGVs may be driven by the P/LP CPG variants and not the additional pFGVs.

4.The KOREA1K data were not processed in the exact manner as the SMC cohort – Methods state that VCFs generated in hg38 were downloaded and lifted over to hg19. To test for enrichment, these data should be fully processed in the same manner as the SMC cohort. There should be at least one control cohort with identical processing to ensure robustness of results.

5.In addition, for CPG pFGV burden in TARGET the cohort was not compared to a "healthy" control cohort. The adult TCGA data were not processed in the same manner and therefore this is not an appropriate comparison. It is also unclear why TARGET was compared to TCGA but the SMC cohort was not.

6.The survival analyses are still based on dichotomized data "high" vs. "low". The explanation provided in Response 2.8 "we chose to dichotomize in this instance due to the heterogeneous nature of variant impacts. This variability arises from differences in gene function and mutation severity" is not resonating. While it is true that there will be variability in gene function and severity, the reason to think that dichotomizing the data would resolve this issue is not clear. Response 2.8 reports that when the pFGV burden was used as a continuous value, the result was not significant.

7.Response 2-6. While the code is now on GitHub, without companion data, it is not possible to evaluate or reproduce results/figures.

8.The "development" and "internal validation" groups used for the C-index analysis with bootstrapping are not defined. Please clarify in the Methods.

9.Findings in the paper are not set in accurate context leading to overstatement of novelty of results. This should be addressed and clarified throughout the paper.

For example, Discussion (first para) states that "Germline investigations have only focused on their correlation with cancer risk". This is not accurate, the influence of germline variants on tumor phenotype in neuroblastoma is firmly established. There are multiple papers demonstrating that germline variants in neuroblastoma are not only associated with tumor initiation/risk but are also key to maintenance or progression of the malignant phenotype, most notably germline ALK mutations (Mosse et al, Nature 2008 and many follow-up papers as well as clinical trials targeting mutant ALK), but also common germline variants (e.g. Wang et al, Nature 2011; Diskin et al, Nat Genet 2012; Bosse et al, Cancer Res 2012; Cimmino et al, Int J Cancer 2018) and more recently rare pathogenic variants (e.g. Randall et al, JNCI 2023). This should be clarified to put the work in proper context.

Similarly, the Discussion states that “While recent studies have reported the prevalence and prognostic value of P/LP variants in CPGs, the prevalence of P/LP variants was lower than expected, and clinical significance was only reported in OS” and cites Kim et al, MedRxiv 2023, now published in JNCI. This statement is not accurate. The prevalence reported in this paper was actually higher than expected based on prior publications in childhood cancers. Kim et al was also the first to report the that patients harboring P/LP variants in CPGs have worse outcome. This is not appropriately discussed in the manuscript. OS is the most critical endpoint as unfortunately nearly all high-risk neuroblastoma patients who relapse still ultimately succumb to the disease. In addition, a correlative study of clinical and tumor biologic variables was included in Kim et al, similar to presented here. Finally, it should be clearly noted that the TARGET exomes analyzed in this manuscript are a subset of the patients studied in Kim et al. JNCI.

As another example, the Discussion states “This finding is significant because the majority of patients do not have MYCN amplification, and thus, a strong prognostic factor for them has been elusive until now.” When referring to the report of pFGVs and PFS. However, this is not accurate. A strong prognostic factor in the MYCN non-amplified set is the presence of segmental copy number alterations (CNAs) (Attiyeh et a,l NEJM 2008; Janoueix-Lerosey et al, JCO 2009; Schleiermacher et al, BJC Genetics and Genomics 2011). This is clearly established and should be referenced, at the very least the authors should not imply that this manuscript is the first prognostic factor identified for this subset of patients.

Overall, the work should be placed in proper context for the reader.

MINOR:

1.TARGET reports EFS, not PFS. These can be different, and this should be clarified in the manuscript as it may affect interpretation of results. Portions of the text referring to PFS in the TARGET cohort should be changed to EFS (the event may or may not be disease progression).

2.Neuroblastoma treatment has evolved over the years. It would be interesting to know the treatment era of patients In the SMC cohort compared to TARGET.

3.Change TARGETR to TARGET in Figure 2 title.

4.“Germeline” needs to be changed to “Germline” in Figure 6.

5.“Germeline” needs to be changed to “Germline” in Extended Data Figure 5.

Responses to reviewer's comments on the manuscript submitted by Seo et al., "Germline Functional Variants Contribute to Somatic Mutation and Outcomes in Neuroblastoma" (Manuscript ID: NCOMMS-23-27293B)

We express our appreciation to the reviewers for their thorough evaluation and insightful contributions to our manuscript. Their detailed critiques and beneficial recommendations have significantly contributed to the refinement of our work. We have taken all their remarks into account and have made the necessary amendments to our manuscript in line with their suggestions.

Enclosed, please find our point-by-point response to the reviewers' feedback. For ease of review, the original comments from the reviewers are presented in bold, while our corresponding responses are in blue. We have articulated the justifications for the amendments implemented, and where our views diverged, we have provided a comprehensive justification. Changes made to the manuscript are clearly marked by indicating the page and line numbers and are also highlighted to facilitate quick identification. In instances where we did not incorporate a particular recommendation from a reviewer, we have furnished a thorough explanation for our stance.

We trust that the modifications we have made fulfill the expectations of the reviewers, and we are receptive to any additional guidance or commentary they may wish to provide.

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REVIEWER COMMENTS

Reviewer #1

General comments: The authors have addressed all the questions raised in the previous review except for a minor comment related to the abstract "Abstract—all descriptive without the statics to justify the importance". The authors opted not to revise to keep within the 150-word limit. **I would suggest that statements in abstract listed below leaves the impression of lack of scientific rigor of the study and the authors should consider revising.** Some part of the abstract (e.g. you do not need to spell out the full TARGET in the abstract to save words) can be made more succinct so that odds ratio, p values can be presented to quantify the significance of their findings.
"The enrichment of pFGVs in cancer predisposition genes was evident in neuroblastoma compared to that in healthy and adult-onset cancer populations, and their presence had prognostic significance in neuroblastoma. The combination of germline and clinical risk factors improves survival predictions."

Response: Thank you for your positive and constructive feedback. The insights from the previous review were invaluable in refining our manuscript. Following the reviewer's suggestion, we have incorporated statistical metrics in the abstract to better quantify the significance of our findings.

Abstract: "*The enrichment of pFGVs in cancer predisposition genes was borderline significant when compared to healthy populations (SMC, P = 0.077, P = 0.077; Odds Ratio, 1.45; 95% CI, 0.94−2.21) and significantly more pronounced against adult-onset cancers (TARGET, P = 0.016; Odds Ratio, 2.13; 95% CI, 1.10−3.91). Additionally, the presence of these variants proved to have prognostic significance in neuroblastoma (log-rank P < 0.001), and combining germline with clinical risk factors notably improved survival predictions.*"

Reviewer #3

General comments: I think the authors have done a very thorough and detailed analysis of germline variants in cancer predisposition genes in patients with neuroblastoma. They infer that these germline functional variants may contribute both to somatic mutations in tumors and to patient outcomes. The authors have addressed all the comments and concerns, and I have no additional comments.

Response: We appreciate the reviewer's positive evaluation of our analysis on germline variants in neuroblastoma patients. It's gratifying to know our responses to previous comments have met the reviewer’s satisfaction. Thank you for your constructive feedback throughout this review process.

Reviewer #4: Expert in neuroblastoma genomics; replaces Reviewer #2

General comment: Seo and colleagues perform germline whole exome sequencing in a cohort of 125 neuroblastoma patients from Korea, termed the SMC cohort. Importantly, this adds an Asian cohort to the two large neuroblastoma germline studies already published to date (Bonfiglio et al, eBioMedicine 2022: n=664 Italian patients and Kim et al, JNCI 2023: n=786 patients from North America; this is an extended version of the TARGET project). The authors identify putative functional germline variants (pFGVs; predicted LOF and missense variants present in < 1% of ExAC) and test for association with tumor mutation burden, clinical variables, and outcomes as well as enrichment compared to controls and adult cancers. They report a positive correlation between germline pFGV burden and tumor mutation burden in their cohort. This is replicated using exome sequencing data (n=220) from the TARGET project. No association was observed between pFGVs and clinical variables in the SMC cohort, consistent with prior reports by Bonfiglio and Kim. A statistically significant association was observed between pFGV burden and progression free survival (PFS) in the SMC cohort and overall survival (OS) in the TARGET data when pFGV burden was dichotomized using the mean burden as a cutoff; however, this did not remain significant in an analysis where pFGV burden was evaluated as a continuous variable (Response 2.8). Finally, the authors perform a focused study of 109 cancer predisposition genes (CPGs). There was a trend toward enrichment of pFGVs in the SMC cohort compared to KOREA1K controls; however, this only became statistically significant when further restricted to pathogenic or likely pathogenic (P/LP) variants in CPGs according to ACMG criteria. The TARGET cohort was not compared to a "healthy" control cohort, but rather was compared to adult cancers in TCGA. This analysis showed an enrichment of pFGVs in TARGET vs. TCGA. However, it is noted that neither the control cohort (KOREA1K) nor the adult cancer cohort (TCGA) were processed in the identical manner as the neuroblastoma patient data. Finally, the authors report results of various analyses investigating the role of pFGVs in patient outcomes, including survival. They conclude the pFGVs are prognostic in neuroblastoma, particularly in the MYCN non-amplified subset.

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results (Kim et al PLOS One, 2023). The finding that pFGV burden associates with worse outcome is also of interest. However, several portions of the results presented do not control for the influence of known P/LP variants in CPGs, which have been demonstrated to associate with worse outcomes in a study of 786 neuroblastoma cases, including the TARGET cohort (Kim et al, JNCI 2023; referenced as Kim et al, MedRxiv 2023 in the initial submission of this manuscript). The analyses also rely on dichotomized data rather than considering pFGV as a continuous variable. Taken together, this raises questions regarding the true contribution of pFGVs in neuroblastoma beyond P/LP variants in CPGs. Overall, the manuscript is well written and presents some interesting results. **However, the enrichment and survival portions of the manuscript require further clarification to discern the true contribution of pFGVs.** In addition, **findings in the paper are not set in accurate context leading to overstatement of novelty of results**. This should be addressed and clarified throughout the paper.

Response: We are immensely grateful for the detailed and constructive feedback provided by Reviewer #4. The depth of expertise in neuroblastoma genomics that the reviewer brings is clearly evident, and we deeply value the insights shared with us. The concerns regarding the methodological differences in variant calling between our neuroblastoma cases and the control group, as well as the queries about the validity of our enrichment analysis for potentially functional germline variants (pFGVs) in cancer predisposition genes (CPGs), are considered of utmost importance. Additionally, the reviewer has highlighted areas within our discussion that necessitate modification. We have thoughtfully reviewed these points and have integrated the suggested amendments into our revised manuscript. Furthermore, we have taken all other comments into serious consideration and have diligently worked to address each one in our revised manuscript. It is our genuine hope that the updates and the responses we have provided will align with the reviewer's expectations.

MAJOR:

Comments 4-1: Figure 2 – the somatic mutation burden for TARGET exomes presented in panels A and B do not appear to agree. based on a log 10 transformation. The base of the logarithm in Panel B may be mislabeled, or there is a larger issue that needs to be addressed.

Response 4-1: Thank you for pointing out the discrepancy between the representations in Fig. 2, Panels a and b. Upon review, we acknowledge the confusion caused by the different treatments of the y-axis (somatic mutation burden) in these panels. To clarify, the y-axis in Fig. 2a represents the somatic mutational burden without logarithmic transformation, while in Fig 2b, we applied a log10 transformation to the somatic mutation burden **for visualization purposes**. This transformation was implemented to address the wide distribution of somatic mutational burden and to enhance the clarity

and consistence of the data presentation (as Fig. 1), especially given the broad range of values.

We also emphasize that the use of **Spearman's correlation** for the statistical analysis in Fig 2 was chosen specifically for its non-parametric nature, which allows for the assessment of correlation without assuming a normal distribution of the data. Therefore, **the application of a $\log_{10}$ transformation for visualization does not influence the correlation coefficient or the p-value derived from Spearman's correlation**.

Comments 4-2: Figure 2D – why does this only include TARGET exomes? What about the SMC exomes? Are results consistent?

Response 4-2: In response to the reviewer's insightful inquiry concerning Fig. 2d, we wish to elucidate our methodology further. Our intent was to mitigate potential biases stemming from ethnic differences when comparing the associations between germline variants and somatic mutations in neuroblastoma patients to those in adult-onset cancer cohorts. As delineated in the methods section (TCGA dataset), our analysis in Fig. 2d specifically targeted individuals of **white ethnicity** within both the TARGET and TCGA pan-cancer cohorts. This deliberate focus was crucial for establishing a consistent comparison basis, thereby minimizing the influence of ethnic diversity on our analysis.

Concerning the SMC exomes, we embraced different population filtering strategy, employing the KRGDB (the Korean Reference Genome Database)[1] 1100 <1% threshold which is specific to Korean. This particular criterion was not applied to the TARGET/TCGA cohorts due to the complex challenge of precisely defining individual ethnic backgrounds in these datasets, hindering the application of analogous filtering criteria.

Addressing the point raised by the reviewers regarding the SMC cohort, we observed a Spearman rho correlation of 0.16. This correlation is in line with the patterns noted in the 19−29 and 30−39 age groups within the TCGA cohort. It is imperative to recognize that ethnic composition and differences in population filtering approaches across cohorts necessitate careful consideration when interpreting these results.

We acknowledge that the description and legends associated with Fig. 2d were not updated to explicitly indicate our focused comparison on white ethnicities. This oversight has been rectified to ensure clarity in our comparative analysis and to avoid any potential misunderstanding regarding our study's scope and the populations analyzed.

In response to the reviewer's comment, we have now amended our manuscript and legend of Fig. 2d.

Page 5, lines 131−133: "*Finally, we analyzed the association between germline variant burden and somatic mutational burden across all age groups at diagnosis. This analysis included patients with neuroblastoma from the* ***white ethnicity subgroup within the TARGET cohort****.*"

Fig. 2: 2d, Trends in Spearman's correlation coefficient and confidence intervals between germline variant burden and somatic mutational burden across age groups at diagnosis in the TARGET and TCGA (**restricted to white ethnicity only**).

Comments 4-3: The results section labeled "pFGVs in CPGs are enriched in neuroblastoma" is misleading. The abstract and results state there was an enrichment of pFGVs in CPGs in SMC cohort compared to KOREA1K. However, the p-value is not statistically significant. **This should be reworded, including the result heading and abstract**. In fact, it was only when restricting to P/LP variants in CPGs according to ACMG guidelines that a significant enrichment was observed. This finding has been reported in a cohort of 786 neuroblastoma patients, including the TARGET exomes (Kim et al, JNCI – referenced in the original manuscript as Kim et al, MedRxiv 2023). **While it is important that the finding of CPG P/LP variant enrichment was replicated here in the SMC cohort, it seems plausible that the trend toward significance for the broader set of pFGVs may be driven by the P/LP CPG variants and not the additional pFGVs.**

Response 4-3: We are thankful for the reviewer's astute comments and the highlight of areas requiring enhanced clarity in the presentation of our results. Following the reviewer's constructive feedback, we have updated the title of the results section, along with the related summaries in both the abstract and the main body of the text. These revisions more precisely convey our findings and their statistical significance.

Abstract: "*The enrichment of pFGVs in cancer predisposition genes was borderline significant when compared to healthy populations (SMC, P = 0.077, P = 0.077; Odds Ratio, 1.45; 95% CI, 0.94−2.21) and significantly more pronounced against adult-onset cancers (TARGET, P = 0.016; Odds Ratio, 2.13; 95% CI, 1.10−3.91). Additionally, the presence of these variants proved to have prognostic significance in neuroblastoma (log-rank P < 0.001), and combining germline with clinical risk factors notably improved survival predictions.*"

Page 5, lines 149: We have revised the section title from "pFGVs in CPGs are enriched in neuroblastoma" to "Enrichment analysis of pFGVs in CPGs of neuroblastoma".

Comments 4-4: The KOREA1K data were not processed in the exact manner as the SMC cohort – Methods state that VCFs generated in hg38 were downloaded and lifted over to hg19. To test for enrichment, these data should be fully processed in the same manner as the SMC cohort. There should be at least one control cohort with identical processing to ensure robustness of results.

Response 4-4: We appreciate the opportunity to address the concerns raised regarding the methodological differences between the KOREA1K and SMC cohorts in our study. Our initial intention was to process the KOREA1K data in a manner identical to that of the SMC cohort, beginning from raw FASTQ files. Unfortunately, due to restrictions on data access, we were compelled to work with pre-processed VCF files, which involved lifting over from hg38 to hg19. Despite this, we maintained a high standard of data integrity by closely following GATK best practices for variant calling similar to the approach used for the KOREA1K data, aside from the reference genome alignment.
Aware of the potential discrepancies introduced by the lift-over process, we implemented stringent quality control measures. This careful approach, supported by existing literature[2,3], significantly minimizes the discordance rates associated with genomic data conversion. Furthermore, our decision to compare the qualitative presence of pFGVs in CPGs, rather than total variant burden, was a deliberate and this focus on pFGVs might help to minimize the introduction of noise from differential processing.

The choice of the KOREA1K dataset[4] as a control was informed by the balance between methodological consistency and the representativeness of the control cohort. Given its composition of Korean-specific individuals without pediatric cancer or other rare diseases, the KOREA1K dataset offers a contextually relevant comparison for our SMC cohort, ensuring the demographic specificity of our findings.

Moreover, the practice of utilizing control cohorts processed through different methodologies is not uncommon in the genomic research field. For example, the study referenced in the general comments by the reviewer **utilized aggregated variant data instead of processing raw data directly**. Specifically, the study by Kim et al. compared neuroblastoma cases—derived from a combination of whole genome sequencing (WGS, n = 134), whole exome sequencing (WES, n = 222), and panel sequencing of 166 genes (n = 489)—with WES data from the PMBB and the gnomAD database. While the neuroblastoma (case) and PMBB (control) data were processed in the same manner, the sequencing depth and targets were indeed different. Moreover, **the gnomAD database compiles variants from a wide range of general population studies and was not processed uniformly**. Similarly, the study by Bonfiglio et al(eBioMedicine, 2023)[5]. **also relied on aggregated variant data**, not processing case and control data identically. Another example is the work by Akhavanfard et al. (Nature Communications, 2020)[6], which compared PV/LPV in CPGs with the non-TCGA ExAC dataset, undergoing a distinct

processing approach compared to their case data.

However, acknowledging the reviewer's concern, we plan to include this discussion in the limitations section of our manuscript, underscoring that interpretations of germline variant burden and pFGVs in CPGs necessitate caution. The variance in methodology across cohorts introduces complexities in making direct comparisons, a critical point we aim to transparently communicate.

Page 9, lines 265−271: "*Furthermore, our control cohorts were not subjected to the same experimental conditions or variant calling processes as the case cohorts, as they relied on pre-processed variant data. This introduces a layer of complexity that might affect the comparability of our findings. The total count of germline variants and the identification of pFGVs in CPGs identified could have been affected by the specific experimental design and variant filtering processes, which varied across cohorts. Consequently, interpretations of the germline variant burden and the presence of pFGVs in CPGs should be approached with caution at an individual level, and this variance in methodology complicates direct comparisons between cohorts.*"

Comments 4-5: In addition, for CPG pFGV burden in TARGET the cohort was not compared to a "healthy" control cohort. The adult TCGA data were not processed in the same manner and therefore this is not an appropriate comparison. It is also unclear why TARGET was compared to TCGA but the SMC cohort was not.

Response 4-5: We appreciate the opportunity to address the points raised in your comments, and we hope that our responses, including those in **response 4-4**, have helped to alleviate some concerns regarding our approach and conclusions.
Our enrichment analysis aimed to delve into the prevalence of pFGVs in CPGs, focusing on the comparison between neuroblastoma cases in the TARGET cohort and a broad spectrum of adult cancers represented in the TCGA database. This comparison was motivated by our observations of an inverse relationship between germline variant and somatic mutational burdens between these cohorts. Our analysis aimed to fill the gaps not addressed by previous comparisons with "healthy" control cohorts, which is a well-trodden area of research. Our choice to examine these differences was also driven by the unique research questions we sought to answer, focusing on uncovering new insights by comparing these distinct patient populations. We selected the TCGA database for its diversity, containing data from 31 different solid tumor types. This variety offers an unbiased control group, helping to mitigate biases associated with focusing on a single cancer type or age-specific dataset.

We acknowledge the importance of methodological consistency and the ideal scenario of matching controls and cases in processing methods to enhance the reliability of our findings. However, the primary objective of our comparative analysis was to prioritize the representativeness and relevance of the cohorts within the practical limitations related to data availability and the computational demands of processing the TCGA dataset, which includes over 10,000 samples.

In our previous response, we mentioned that it is not uncommon for studies to utilize pre-processed variant datasets instead of raw data. Another relevant example for this can be found in the work by Loveday et al., published in Annals of Oncology (2022)[7], which investigates germline variants in predisposition genes among breast cancer patients compared to healthy controls. For their control group, Loveday et al. utilized summary data from gnomAD, a decision that underscores the feasibility of using large, pre-processed datasets for control groups in genetic studies.

As for the specific comparison between the TARGET and TCGA datasets and not including SMC, we have previously addressed the critical role of ethnicity in our comparative analyses in **response 4-2**. We focused on comparing white patients in the TARGET cohort with white samples in TCGA, aligning them not only by their comparable ethnic backgrounds but also by utilizing a consistent rare variant filtering for identifying pFGVs. Given that TCGA includes a relatively small number of Asian individuals, which does not adequately represent specific subgroups like East or South Asians, and the impracticality of applying the same Korean data filters from the SMC cohort to TCGA, we opted not to use these samples in our comparison.

We hope this explanation relieves the reviewer's concerns.

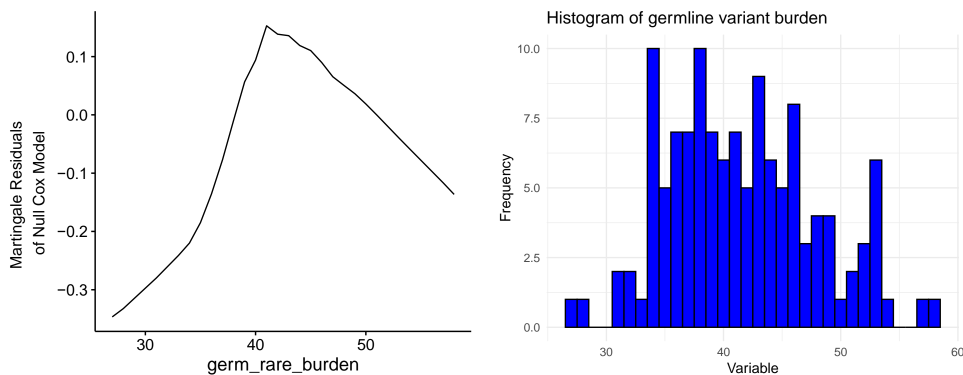
Comments 4-6: The survival analyses are still based on dichotomized data "high" vs. "low". The explanation provided in Response 2.8 "we chose to dichotomize in this instance due to the heterogeneous nature of variant impacts. This variability arises from differences in gene function and mutation severity" is not resonating. While it is true that there will be variability in gene function and severity, **the reason to think that dichotomizing the data would resolve this issue is not clear**. Response 2.8 reports that when the pFGV burden was used as a continuous value, the result was not significant.

Response 4-6: In addressing the concerns regarding our use of dichotomized data for survival analyses, we appreciate the opportunity to further elucidate it. The heterogeneity inherent in gene function and the complex nature of genetic variations may manifest as **non-linear relationships between the genetic variables and clinical outcomes**. Such non-linearities may not conform to the assumptions underlying

many statistical tests, which can obscure significant relationships when genetic variables are treated as continuous.

To address this, we have conducted several additional analyses:

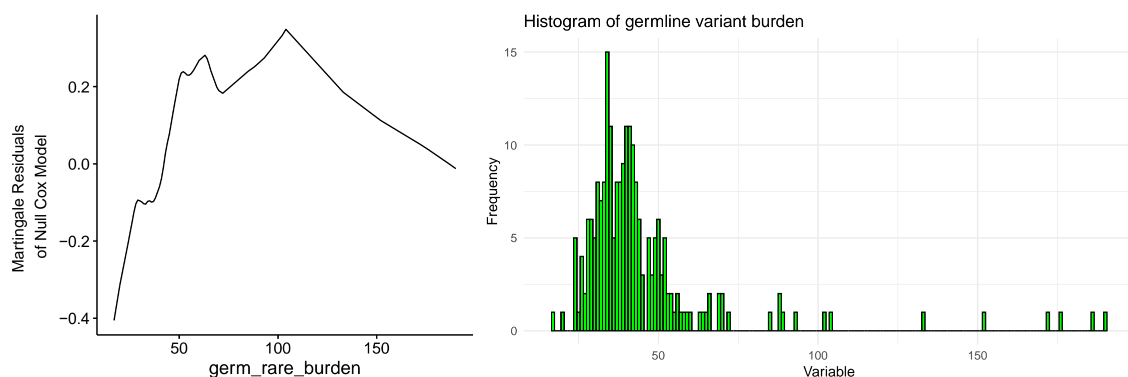
1. Plotting Martingale residuals from a null **Cox proportional hazards model against the germline rare burden revealed a non-linear relationship**, where the model underestimates the risk at higher burden levels.



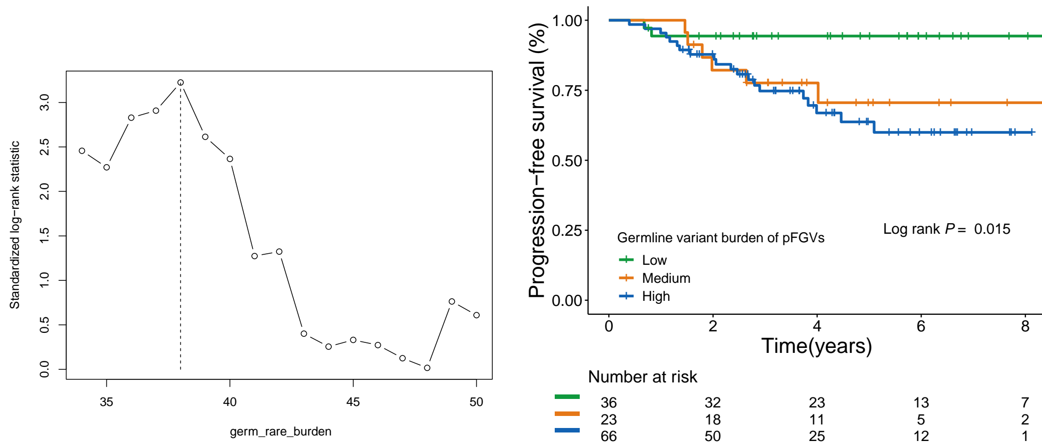
   These non-linearities may lead to a loss of significant findings when germline rare burden is treated as a continuous variable in a Cox proportional hazards model, particularly in the presence of a ceiling effect as noted in our data.

2. When considering only patients with a germline mutation burden lower than the average (41), as depicted in the figure above, where a linear relationship is observed near 41, treating the mutation burden as a continuous variable revealed a significant prognostic factor (HR 1.57, 95% CI, 1.05−2.37, P = 0.029). However, this analysis excluded some patients.

3. For the TARGET cohort, a similar non-linear relationship between germline variant burden and survival outcome was observed. However, the data distribution was skewed, as indicated by the histogram analysis, leading to mostly linear relationship. By applying a log transformation to the germline variant burden (for skewed data), we uncovered prognostic significance for overall survival (HR 1.53, 95% CI, 1.03−2.28 P = 0.034), which was not apparent without this transformation.



4. A sensitivity analysis using standardized log-rank statistics identified two peak cutoff points (38 and 41), allowing us to stratify patients into three distinct groups. The low burden group exhibited higher survival rates.



5. We demonstrated the robustness of our findings across different categorization methods by calculating log-rank *P*-values for all possible cutoff points.

Cut-off	*P* value (log-rank)	Cut-off	*P* value (log-rank)
28	0.8759	43	0.1832
29	0.5548	44	0.6882
30	0.5548	45	0.7967
31	0.5548	46	0.7368
32	0.3696	47	0.7794
33	0.2537	48	0.8974
34	0.198	49	0.9866
35	0.0212	50	0.4429
36	0.0301	51	0.5359
37	0.0065	52	0.7505
38	0.0047	53	0.9612
39	0.0015	54	0.4441
40	0.0095	55	0.4885
41	0.0181	56	0.4885

42	0.2017	57	0.4885

In summary, we adopted a more comprehensive approach that accounts for potential non-linearity to establish a more robust association between germline variant burden and prognosis. **It suggests that the relationship between germline variant burden and survival may be better captured by a non-linear term or by categorizing germline variant burden into meaningful groups.**

In conclusion, it is challenging to conduct survival analyses assuming a simple linear relationship between germline variant burden and outcome, due to the diverse effects of genetic functional abnormalities and the distinct conditions and characteristics of each cohort. Therefore, we have decided that it is more appropriate to continue with our consistent and straightforward approach to illustrate the relationship between germline variant burden and outcome. Our current approach retains the use of dichotomization, acknowledging that each cohort requires its unique statistical considerations.

Comments 4-7: While the code is now on GitHub, without companion data, it is not possible to evaluate or reproduce results/figures.

Response 4-7: We sincerely appreciate your attention to the detail regarding the provision of our code on GitHub. Our intent with making the code available was primarily to demonstrate our analytical processes.

However, the upload of the TARGET dataset to a public repository is prohibited for us, as we do not hold ownership or the right to distribute this data due to strict data usage agreements and privacy considerations. Regarding our own SMC dataset, privacy concerns and institutional guidelines prevent us from sharing detailed patient clinical information publicly.

In an effort to balance transparency with these constraints, we will upload two anonymized VCF files with sex chromosome information removed. This partial disclosure is intended to offer insight into our variant counting process while respecting the privacy and confidentiality of the data subjects involved. Please note, while we strive to make our research as open and accessible as possible, the distribution of complete datasets, especially those including sensitive clinical information, is bound by ethical and legal restrictions. We have made FASTQ files available through SRA, but access to the associated clinical information would necessitate institutional approval.

Comments 4-8: The "development" and "internal validation" groups used for the C-index analysis with bootstrapping are not defined. Please clarify in the Methods.

Response 4-8: Thank the reviewer for valuable feedback. In accordance with the reviewer's suggestion,

we have updated the Methods section to include a detailed description of the development and internal validation cohorts utilized in our C-index analysis with bootstrapping.

Page 13, lines 390−393: "*For internal validation of our predictive model, we performed permutation testing over 500 iterations, randomly dividing the dataset into development (60%) and internal validation (40%) sets for each cycle. The model's discriminatory power was quantitatively assessed using Harrell's C-index, conducted with 500 bootstrap replicates to ensure robustness.*"

Comments 4-9: Findings in the paper are not set in accurate context leading to overstatement of novelty of results. This should be addressed and clarified throughout the paper.

For example, Discussion (first para) states that "Germline investigations have only focused on their correlation with cancer risk". This is not accurate, the influence of germline variants on tumor phenotype in neuroblastoma is firmly established. **There are multiple papers demonstrating that germline variants in neuroblastoma are not only associated with tumor initiation/risk but are also key to maintenance or progression of the malignant phenotype**, most notably germline ALK mutations (Mosse et al, Nature 2008 and many follow-up papers as well as clinical trials targeting mutant ALK), but also common germline variants (e.g. Wang et al, Nature 2011; Diskin et al, Nat Genet 2012; Bosse et al, Cancer Res 2012; Cimmino et al, Int J Cancer 2018) and more recently rare pathogenic variants (e.g. Randall et al, JNCI 2023). This should be clarified to put the work in proper context.

Similarly, the Discussion states that "While recent studies have reported the prevalence and prognostic value of P/LP variants in CPGs, the prevalence of P/LP variants was lower than expected, and clinical significance was only reported in OS" and cites Kim et al, MedRxiv 2023, now published in JNCI. This statement is not accurate. **The prevalence reported in this paper was actually higher than expected based on prior publications in childhood cancers. Kim et al was also the first to report the that patients harboring P/LP variants in CPGs have worse outcome.** This is not appropriately discussed in the manuscript. OS is the most critical endpoint as unfortunately nearly all high-risk neuroblastoma patients who relapse still ultimately succumb to the disease. In addition, a correlative study of clinical and tumor biologic variables was included in Kim et al, similar to presented here. **Finally, it should be clearly noted that the TARGET exomes analyzed in this manuscript are a subset of the patients studied in Kim et al. JNCI.**

As another example, the Discussion states "**This finding is significant because the majority of patients do not have MYCN amplification, and thus, a strong prognostic factor for them has been**

elusive until now." When referring to the report of pFGVs and PFS. However, this is not accurate. A strong prognostic factor in the MYCN non-amplified set is the presence of segmental copy number alterations (CNAs) (Attiyeh et a,l NEJM 2008; Janoueix-Lerosey et al, JCO 2009; Schleiermacher et al, BJC Genetics and Genomics 2011). This is clearly established and should be referenced, at the very least the authors should not imply that this manuscript is the first prognostic factor identified for this subset of patients.

Overall, the work should be placed in proper context for the reader.

Response 4-9: We express our profound gratitude for the reviewer's expertise and the insightful feedback provided. The reviewer has brought to our attention several references that we had not fully considered, and we concur with most of the points raised. We realize that parts of our discussion may have contained ambiguities that could lead to misunderstandings. Accordingly, we have revisited the sections emphasized by the reviewer, incorporating the suggested references into our revised text and citation list. Furthermore, we have updated the citation of Kim et al.'s work to reflect its publication status as rightly pointed out by the reviewer.

Page 7, lines 210−214: *"However, it is becoming increasingly clear that germline variants, inherent to each patient's genetic makeup, can significantly shape tumor characteristics. Mounting evidence underscores the significance of germline variants, extending beyond cancer susceptibility to influence tumor progression and phenotype. Our work builds on this foundation, focusing on the comprehensive analysis of rare germline variants and their broader implication in tumor biology and patient outcomes."*

Page 8, lines 238−241: *"The study by Kim et al., which includes analyses from the TARGET dataset that our research also examines, highlights the prevalence and potential prognostic implications of P/LP variants in CPGs.[8-10] However, it is important to recognize that our understanding of the role of these variants across a broader patient population remains limited."*

Page 9, lines 248−251: *"Furthermore, we showed that pFGVs in CPGs serve as critical determinants of OS in patients without the strongest somatic driver alterations (MYCN amplification) in the both SMC and TARGET cohorts. Overall, our study may expand the definition of pathogenicity and highlights the significance of the identified pFGVs."*

Page 9, line 280−282: "*Additionally, we have broadened our understanding of pathogenic variants in CPGs, encompassing aspects beyond disease predisposition.*"

MINOR:

Comments 4-10: TARGET reports EFS, not PFS. These can be different, and this should be clarified in the manuscript as it may affect interpretation of results. Portions of the text referring to PFS in the TARGET cohort should be changed to EFS (the event may or may not be disease progression).

Response 4-10: We appreciate the reviewer's attention to detail regarding our manuscript. The reviewer correctly points out that the TARGET database reports EFS rather than PFS. Initially, our goal was to compare the relationship between specific variables and PFS in both the SMC and TARGET cohorts. **We had derived a PFS measurement for TARGET by categorizing cases with relapse or progression as the first event.** This strategy was based on the NCBI's TARGET variable guide definitions, which consider relapse, progressive disease, secondary malignancy, or death as events, the latter two acting as competing risks. **However, it became apparent that in the TARGET dataset, not all events were explicitly categorized, which led to the possibility of misclassification between relapse/progression and other events such as death.** This ambiguity prevents a reliable calculation of PFS in the TARGET cohort.

Therefore, in alignment with the reviewer's comments and precision required for calculating PFS, **we have removed the PFS analysis from the TARGET data in our manuscript.** We also thought it pertinent to share with the reviewer that our survival analysis of EFS in relation to variables such as germline variant burden or the presence of pFGVs in CPGs within the TARGET cohort did not reach statistical significance (log-rank *P* value = 0.112, 0.167, respectively). We appreciate the reviewer's guidance in enhancing the accuracy of our results.

Comments 4-11: Neuroblastoma treatment has evolved over the years. It would be interesting to know the treatment era of patients In the SMC cohort compared to TARGET.

Response 4-11: In response to the reviewer's insightful suggestion in Comments 4-11, we have added the following paragraphs to the discussion section. We very much appreciate this helpful comment.

Page 9, lines 252−261: "*Neuroblastoma treatment strategies have considerably evolved over time, reflecting advances in medical research and clinical practice. It is essential to contextualize our findings within the treatment era of the patient cohorts studied. The TARGET cohort, comprising exclusively high-risk patients, experienced a wide variety of high-risk treatment protocols. These included different induction regimens, the use of high-dose chemotherapy, variations in both the chemotherapy regimens and the number of high-dose chemotherapy cycles, adjustments in radiation therapy doses, and the*

introduction of anti-GD2 maintenance therapy. In contrast, the SMC cohort, which included patients from all clinical risk groups, could not utilize anti-GD2 therapy. Instead, for high-risk patients, it adopted the implementation of intensified tandem high-dose chemotherapy and high-dose MIBG treatment. Despite these differences and changes in treatment paradigms, the prognostic value of germline variants remains evident."

Comments 4-12: Change TARGETR to TARGET in Figure 2 title.

Response 4-12: We appreciate this catch. The correction has been made to change "TARGETR" to "TARGET" in the title of Figure

Comments 4-13: "Germeline" needs to be changed to "Germline" in Figure 6.

Response 4-13: Thank you for pointing out the typographical error. The word "Germeline" has been corrected to "Germline" in the title of Figure 6.

Comments 4-14: "Germeline" needs to be changed to "Germline" in Extended Data Figure 5.

Response 4-14: We have also addressed the similar typo in Extended Data Figure 5, updating "Germeline" to the correct spelling, "Germline."

We hope that we have clearly addressed all your comments and have revised appropriate discussion in the paper.

Reference

1 Jung, K. S. *et al.* KRGDB: the large-scale variant database of 1722 Koreans based on whole genome sequencing. *Database* **2020**, doi:10.1093/database/baz146 (2020).

2 Pan, B. *et al.* Similarities and differences between variants called with human reference genome HG19 or HG38. *BMC Bioinformatics* **20**, 101, doi:10.1186/s12859-019-2620-0 (2019).

3 Ormond, C., Ryan, N. M., Corvin, A. & Heron, E. A. Converting single nucleotide variants between genome builds: from cautionary tale to solution. *Briefings in Bioinformatics* **22**, doi:10.1093/bib/bbab069 (2021).

4 Jeon, S. *et al.* Korean Genome Project: 1094 Korean personal genomes with clinical information. *Sci Adv* **6**, eaaz7835, doi:10.1126/sciadv.aaz7835 (2020).

5 Bonfiglio, F. *et al.* Inherited rare variants in homologous recombination and neurodevelopmental genes are associated with increased risk of neuroblastoma. *EBioMedicine* **87**, 104395, doi:10.1016/j.ebiom.2022.104395 (2023).

6 Akhavanfard, S., Padmanabhan, R., Yehia, L., Cheng, F. & Eng, C. Comprehensive germline genomic profiles of children, adolescents and young adults with solid tumors. *Nat Commun* **11**, 2206, doi:10.1038/s41467-020-16067-1 (2020).

7 Loveday, C. *et al.* Analysis of rare disruptive germline mutations in 2135 enriched BRCA-negative breast cancers excludes additional high-impact susceptibility genes. *Ann Oncol* **33**, 1318-1327, doi:10.1016/j.annonc.2022.09.152 (2022).

8 Akhavanfard, S., Padmanabhan, R., Yehia, L., Cheng, F. & Eng, C. Comprehensive germline genomic profiles of children, adolescents and young adults with solid tumors. *Nature Communications* **11**, 2206, doi:10.1038/s41467-020-16067-1 (2020).

9 Barr, E. K. & Applebaum, M. A. Genetic Predisposition to Neuroblastoma. *Children (Basel)* **5**, doi:10.3390/children5090119 (2018).

10 Kim, J. *et al.* Germline pathogenic variants in neuroblastoma patients are enriched in BARD1 and predict worse survival. *J Natl Cancer Inst* **116**, 149-159, doi:10.1093/jnci/djad183 (2024).

REVIEWERS' COMMENTS

Reviewer #4 (Remarks to the Author):

The authors have addressed all prior comments in a satisfactory manner.