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| **Reviewer comment 1** | In the past year, several papers reporting "human knock-outs" for key genes and loci as detected by NGS have been reported. Often the effect on phenotype is minimal to non-existant. Can the authors interpret such findings in the context of their approach and observations? |
| **Authors' response** | As the reviewer has pointed out correctly, studies focused on identifying and understanding human knockouts have been published recently. A study on British Pakistanis with related parents, identified 781 genes containing rare LoF homozygous variants (PMID: 26940866). They found homozygous LoF variants in recessive Mendelian disease genes in 42 people, however 33 of them did not have the disease phenotype. -ALoFT indeed predicts that 19 variants should lead to disease with a recessive mode of inheritance. However, lack of a discernible phenotype could be due to incomplete penetrance, presence of modifiers or environmental influences. |
| **Changes in text** | A study on British Pakistanis with related parents identified 781 genes containing rare LoF homozygous variants (PMID: 26940866). They found homozygous LoF variants in recessive Mendelian disease genes in 42 people, however 33 of them did not have the disease phenotype. We applied ALoFT to classify these homozygous LoF variants and ALoFT indeed predicts that 19 of them would cause disease- (Supplementary table xx). However, the lack of a discernible phenotype could be due to incomplete penetrance of the mutations or due to modifier effects. The penetrance of some disease mutations are known to be age and sex-dependent (PMID: 19785764). It is well established that there is widespread occurrence of disease variants with reduced penetrance in the general population (PMID: 23820649). While studies in consanguineous populations have been used to identify recessive disease genes (PMID: 25558065, 27435318), absence of disease provides an opportunity to look for modifiers in their genetic background. - |

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| **Reviewer 4 comment 2** | The authors do corroborate some results from the literature in their case studies, but these seem more suggestive of ALoFT's performance rather than conclusive. One concern is that a significant fraction of HGMD variants still receive high benign scores. Some additional analyses would be helpful. |
| **Authors' response** | It is known that HGMD includes erroneous disease annotations and it has been estimated that about 25% of all HGMD annotations (which includes missense and pLoF variants) are erroneous. We have already included this discussion in the manuscript as can be seen on Page 6 paragraph 1. Here is the statement pertaining to this point.<br><br>***In connection with this, it should be noted that the referenced studies are*** |

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| | ***based on diverse methods of identifying variants ranging from targeted panel-based candidate gene studies to whole genome sequencing and disease databases include incorrect disease annotations and common variants -and about 27% of variants were excluded by Bell et al. in their estimate of carrier burden for severe recessive diseases[47].***<br><br>***As requested by the referee we have also done -further analysis on HGMD variants predicted to be benign.*** |
| **Changes in text** | Only 3.7% of HGMD mutations are predicted to be tolerant. Some notable examples of HGMD LoF variants predicted to be tolerant occur in genes such as *FLG, C4orf26 and APOA2*. Filaggrin LoF mutations are linked to susceptibility to atopic dermatitis, a skin condition leading to eczema (PMID: 27659773). Mutations in C4orf26 lead to *Amelogenesis Imperfecta*, a disorder of tooth development. While these mutations are pathogenic, they are not lethal and also known to be genetically heterogeneous (PMID: 20878018). |

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| **Reviewer 4 comment 3** | Also, the authors only briefly comment on confounders like how variations in sequence coverage, variations in variant calling, variations in penetrance, etc. would affect the results. Those seem like important factors to characterize for broad use of this tool by the community. |
| **Authors' response** | All prediction tools work on the assumption that the underlying variant is a true variant. Ascertainment and quality issues of variants are confounders that the user must pay attention in assessing if a variant is real or not. Therefore, estimating the number of deleterious alleles in a healthy individual is a complex problem as has been pointed by the referee. We have described this in detail in the Supplementary Methods in Page 20 (Section 2.3.5) in our previous submission. |
| ~~**Changes in text**~~ | |

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| **Reviewer 5 comment 1** | Since the method takes into account the zygosity of the variant, is there any measure to deal with samples that might be low purity. Unlike looking at the germline, in cancer, samples often have a range of tumor cellularity that affects the somatic changes and the purity might affect the ALoFT scores. I also noticed at the very end in the concluding paragraph that it mentions this works in the context of a diploid model, but that was the only mention of it required to be diploid I could find. I think that would need to be more clear up front as it might limit the usage in tissues that are aneuploid. |
| **Authors' response** | Please note that the method does not take require a diploid model. The zygosity of the variant is not a prediction feature. The point about the diploid model is that most prediction tools describe mutations as pathogenic without telling us if it is likely to be pathogenic in the |

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| | heterozygous or homozygous state. However, the explosion in human sequencing has shown that there are millions of rare variants that have arisen due to rapid population growth. Some of these are deleterious and some are neutral waiting to be fixed by evolution over time. Thus, there is a need to differentiate between pathogenic heterozygous mutations from those that are deleterious only as homozygotes. This issue has been eloquently expounded by Nathan Pearson under the section “What the Kearney scheme gets wrong” where he writes “This problem reflects how such schemes simplistically focus on *variants*, instead of *genotypes*.”<br><br>http://genomena.com/2013/05/22/harmful-by-any-other-name-on-clinical-variant-classification/.<br><br>Our prediction algorithm distinguishes pathogenic heterozygous variants from pathogenic homozygous variants. The prediction is derived from features based on variants in recessive genes and dominant genes. However, we realize what the reviewer is getting at. In the case of somatic mutations, zygosity is not a relevant term. This is the reason we chose to plot overall deleteriousness in Fig 2c where the X-axis is (1-benign score) and is a measure of pathogenicity of the somatic mutation.<br><br>***We have explicitly made this clarification in the revised text as follows.*** |
| **Changes in text** | Due to aneuploidy and clonal heterogeneity of cancer cells, we define an overall measure of deleteriousness as (1-Benign ALoFT score) in the X-axis of Figure 2c. |

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| **Reviewer 5 comment 2** | In samples with a high mutational burden, did you see if the majority of the mutations were pLOF were predicted in one class type or another? Would it be a way to distinguish between drivers and passengers as well particularly in tumors with large mutational burden? |
| **Authors’ response** | We first note that the distinction between dominant and recessive pLoF events, is most appropriate in the context of germline variants. A 'recessive' somatic pLoF mutation, may have as harmful a consequence as 'dominant' somatic pLOF. For example a somatic pLoF may act as the second 'hit' of the 'two-hit' hypothesis.<br><br>In order to distinguish between pLoF events and predicted benign LoF events, we examined the ratio of pLoF mutations to total LoF mutations among our patient samples. We identified a linear relationship between pLoF events and total LoF burden. There is |

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| | no identifiable inflection point between higher mutational burden samples and lower mutational burden samples. This suggests that in general, pLOF events accumulate as a relatively fixed proportion of the total mutational burden.<br>~~**Patrick/Mark, please take a stab at this**~~ |
| **Changes in text** | |

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| **Reviewer 5 comment 3** | In Case Study 3, based on the 20/20 rule, how many of the somatic pLoFs did you identify that would be considered a LOF mutation in the cancer exome set you analyzed? |
| **Authors' response** | We examined genes with associated somatic pLoFs mutations for adherence to the 20/20 rule of Vogelstein et al. 2013. Of the 505 genes with pLoF mutations, 317 meet the 20/20 rule (62.7%), while 188 genes do not. This degree of observed congruency, adds to our confidence in ALoFT's ability to identify meaningful events in carcinogenesis. ~~**Patrick/Yao, please take a stab at this. I remember from our last phone call that Yao had specific thoughts on this.**~~ |
| **Changes in text** | |

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