

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-existent. Can the authors interpret such findings in the context of their approach and observations?
Authors' response	<p>As the reviewer has pointed out correctly, studies focused on identifying and understanding human knockouts have been published recently. A study in Icelandic population identified human knock-outs for 1,171 genes. We have included this in the "Introduction" of the paper in our earlier submission. While this paper describes a catalog of knockouts, no phenotype information is available for the Icelanders. A different 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 that did not associate with the disease phenotype. We applied ALoFT to predict the pathogenicity of the homozygous pLoFs. While ALoFT predicts that 3 of these LoF variants are benign, 19 homozygous variants are indeed predicted to 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. Narasimhan et al (PMID: 26940866) have discussed this observation and also provided a similar rationale in their paper.</p> <p>We have included the ALoFT analysis for the knockouts in the British Pakistani study in the manuscript in Page xx.</p>
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, however carriers of most of these homozygous LoF variants do not have the disease phenotype. We applied ALoFT to classify these homozygous LoF variants. Of the 22 variants for which ALoFT provides predictions, 3 are predicted to be benign. However, 19 homozygous variants are indeed predicted to lead to disease with a recessive mode of inheritance (Supplementary table xx). 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 also known to be age and sex-dependent (PMID: 19785764). 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.
Reviewer 4 comment 1	What is missing in this manuscript is a complete analysis, where the predictions, in the end, are confirmed experimentally.
Authors' response	Experimental validation is beyond the scope of this work. However, we understand the reviewers point about validating predictions. To show the

	robustness of the method, we applied the classifier to known case studies and show that our prediction results agree with published results.
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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	<p>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. This has been discussed in the manuscript on Page 6 paragraph 1. Here is the statement pertaining to this point.</p> <p><i>In connection with this, it should be noted that the referenced studies are 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 diseases⁴⁷.</i></p> <p>To minimize errors due to mistakes in HGMD, we have only used high-confidence mutations labeled as "DM" in HGMD. We have included this detail in the Supplementary Methods in Page xx.</p> <p><i>In addition, as requested by the referee we have also done further analysis on HGMD variants predicted to be benign.</i></p>
Changes in text	<p>Only 0.67% of HGMD mutations are predicted to be tolerant. Of the 119 pLoF autosomal variants in HGMD predicted to be tolerant by ALoFT, the majority of variants arise from Filaggrin, <i>FLG</i>. Examples of tolerant LoF variants include 32 variants from <i>FLG</i>, 4 from <i>APOC2</i> and 3 variants from <i>C4orf26</i>. <i>FLG</i> LoF mutations are linked to susceptibility to atopic dermatitis, a skin condition leading to eczema (PMID: 27659773). Eczema is a complex trait and the resulting phenotypes are highly variable due to the interplay of environmental and genetic factors (PMID: 26385242). Mutations in <i>C4orf26</i> lead to <i>Amelogenesis Imperfecta</i>, a disorder of tooth development. While these mutations are pathogenic, they are not lethal and are also known to be genetically heterogeneous (PMID: 20878018).</p>

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	As requested, we have provided a discussion of confounding factors. This discussion of confounders is available in the Supplementary Methods, on

	<p>page 20 (Section 2.3.5). We agree with the referee, that ascertainment and quality issues of variants are confounders to which the user must pay attention in assessing if a variant is real or not. These factors add complexity to estimating the number of deleterious alleles in a healthy individual.</p>
<p>Reviewer 5 comment 1</p>	<p>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.</p>
<p>Authors' response</p>	<p>Please note that our method does not require a diploid model. The zygosity of a variant is not employed as a prediction feature. ALoFT is a useful prediction tool for a diploid model because other prediction tools that describe mutations as pathogenic without telling us if it is likely to be pathogenic in the heterozygous or homozygous state. However, the explosion in human sequencing has shown that there are millions of rare variants that have risen 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 <i>variants</i>, instead of <i>genotypes</i>."</p> <p>http://genomena.com/2013/05/22/harmful-by-any-other-name-on-clinical-variant-classification/.</p> <p>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.</p> <p><i>We have explicitly made this clarification in the revised text as follows.</i></p>
<p>Changes in</p>	<p>We describe a tool for predicting the impact of pLoF variants. In the context of a diploid model, it may be used to determine whether pLoF variants are likely to</p>

text	lead to recessive or dominant disease. In the context of somatic mutation, the meaning of variant zygosity, or distinguishing between 'dominant' and 'recessive' disease causing mutations, is uncertain/irrelevant. Cancer cells may show aneuploidy and cellular heterogeneity. Therefore, for the evaluation of somatic mutations, we define an overall measure of deleteriousness as (1-Benign ALoFT score) on 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	<p>As suggested by our reviewer, we evaluated ALoFT as a tool for distinguishing driver LoF mutations from passenger LoF mutations among high mutation burden tumor samples. For this evaluation, we used ALoFT to measure the ratio of deleterious LoF mutations to total pLoF mutations for our patient samples. We binned patient samples with at least 1 deleterious LoF mutation according to total mutational burden. Four different intervals were defined based on mutation burden -- less than 100 mutations (N=741 samples), 100 to 1000 mutations (N=202 samples), 1000 to 10000 mutations (N=37 samples), and greater than 10000 mutations (N=18) move to legend or Supplementary Methods. We observe a decrease in deleterious LoF mutations with increasing <u>total</u> mutational burden. All between group differences are significant ($p < 0.01$, Fig xx). <u>However, the ratio of deleterious pLoFs to tolerant pLoFs displayed no significant trend across groups (Supplementary Fig XX). The ratio of deleterious pLoF mutations to tolerant LoF mutations is consistently high across groups (84%).</u></p> <p>In relation to this analysis, we also note that distinction between dominant and recessive pLoF events, is most relevant in the context of germline variants. A 'recessive' somatic pLoF mutation may have similar consequence to a 'dominant' somatic pLOF, for example, as the second 'hit' of the 'two-hit' hypothesis.</p>
Changes in text	-

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 interpret the reviewer's comment as interest in a comparison between our ALoFT predictions, and the 20/20 rule of Vogelstein et. al 2013. The 20/20 rule states that if greater than 20% of somatic mutations associated with a gene are loss of function mutations, that gene may be classified a tumor suppressor. Implicit in the 20/20 rule is that all LoF

	<p>mutations affecting possible tumor suppressor genes are deleterious LoF mutations (not benign LoF mutations). As such, the ability of ALoFT to distinguish between benign and deleterious LoF mutations allows the possibility to refine predictions made by the 20/20 rule.</p> <p>We examined genes that fulfill the the 20/20 rule, and subsequently determined how many LoF mutations associated with these genes are deleterious pLoFs according to ALoFT. Among genes with at least 10 somatic mutations, 107 met the 20/20 rule of Vogelstein et al. For most genes in this list, there is a high ratio of deleterious LoFs to pLoF mutations. Overall, 86% of pLoF mutations affecting these genes are deleterious LoFs (1162/1349). This observation strengthens the case for a gene-level correlation between gene loss of function and tumor suppressor activity in cancer.</p>
Changes in text	<p>To classify genes as tumor suppressors, Vogelstein et al. 2013 proposed a “20/20” rule, whereby a gene is classified as a tumor suppressor if > 20% of the observed mutations in that gene result in loss of function. Among 107 genes that met 20/20 rule criteria, 86% of pLoF mutations affecting these genes were deleterious. In contrast, only xx% of deleterious LoF mutations were found in yy number of genes that do not meet the 20/20 rule (Fig xx). A list of these genes is provided as Supplementary Table XX. This finding strengthens evidence that gene loss of function and tumor suppressor activity are correlated in cancer. In cases where genes display a high somatic pLoF rate but low somatic deleterious LoF rate, ALoFT may be used to refine predictions made by the 20/20 rule through identification of false-positive cases. Perhaps the yellow highlighted sentence not needed.</p>