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A quantitative model to predict pathogenicity of missense variants in the *TP53* gene

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ABSTRACT

Germline pathogenic variants in the *TP53* gene cause Li-Fraumeni Syndrome, a condition that predisposes individuals to a wide range of cancer types. Identification of individuals carrying a *TP53* pathogenic variant is linked to clinical management decisions, such as the avoidance of radiotherapy and use of high intensity screening programs. The aim of this study was to develop an evidence-based quantitative model that integrates independent *in silico* data (Align-GVGD and BayesDel) and somatic to germline ratio (SGR), to assign pathogenicity to every possible missense variant in the *TP53* gene. To do this, a likelihood ratio for pathogenicity (LR) was derived from each component calibrated using reference sets of assumed pathogenic and benign missense variants. A posterior probability of pathogenicity was generated by combining LRs, and algorithm outputs were validated using different approaches. A total of 730 p53 missense variants could be assigned to a clinically interpretable class. The outputs of the model correlated well with existing clinical information, functional data, and ClinVar classifications. In conclusion, these quantitative outputs provide the basis for individualized assessment of cancer risk useful for clinical interpretation. In addition, we propose the value of the novel SGR approach for use within the ACMG/AMP guidelines for variant classification.

Key words: Li-Fraumeni syndrome, *TP53*, variant classification, quantitative

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INTRODUCTION

Li-Fraumeni syndrome (LFS) (MIM# 151623) is a cancer syndrome, inherited in an autosomal dominant pattern, predominantly attributable to pathogenic germline variants in *TP53* (MIM# 191170). Common malignancies in LFS families are soft tissue sarcomas, early-onset breast cancers, brain tumors and adrenocortical carcinomas (Li & Fraumeni, 1969), although this family history is not always apparent due to the high proportion of *de novo* pathogenic variants (Gonzalez et al., 2009; Renaux-Petel et al., 2018). Individuals heterozygous for a *TP53* germline pathogenic variant (hereafter termed carriers) have been previously estimated to have a cumulative cancer incidence of 50% by age 31 and almost 100% by age 70 (Mai et al., 2016). More recently, ascertainment of pathogenic variant carriers through less stringent criteria has resulted in the traditional estimates of penetrance being revised downwards for many breast cancer predisposition genes, including *TP53* (de Andrade et al., 2019; Fortuno, James, & Spurdle, 2018), and extension of the spectrum of cancers linked to the classic LFS phenotype (Rana et al., 2018). The identification of germline *TP53* variants is increasing due to the expanded use of genomic technologies generally in individuals affected by cancer, creating challenges for variant classification and clinical decision-making. There is intense debate on the ideal methods for distinguishing between pathogenic and benign variation, particularly for interpretation of risk in clinical practice (MacArthur et al., 2014). Clinical management of *TP53* carriers include avoidance of radiotherapy to prevent radio-induced cancers (Heymann et al., 2010), and intensive surveillance strategies for early cancer detection (Kratz et al., 2017).

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The *TP53* gene was originally linked to LFS over 20 years ago (Malkin et al., 1990; Srivastava, Zou, Pirollo, Blattner, & Chang, 1990), and is now recognized as one of the most frequently somatically mutated genes across all cancer types (Bouaoun et al., 2016; Muller & Vousden, 2014). The IARC *TP53* Database maintains a comprehensive record of somatic and germline mutations in *TP53* (Bouaoun et al., 2016). The latest version of the database (R19, August 2018) records 29,894 somatic variants (4,527 unique variants), and 457 unique germline variants in 1221 families. In addition, the data includes extensive information on the functional characterization of *TP53* sequence variants, including the comprehensive mutagenesis of all possible amino acid substitutions caused by single nucleotide substitutions and subsequent functional analysis of transactivation activity in yeast (Kato et al., 2003). Further, systematic approaches in human cells to study distinct mutant *TP53* features have been published recently (Giacomelli et al., 2018; Kotler et al., 2018). Despite these resources, there is currently no systematic approach to interpretation of variants in *TP53* that integrates these data sources in a quantitative and probabilistic model. Where similar models have been developed for other genes, such as ENIGMA *BRCA1/2* classification criteria (Spurdle et al., 2012) and InSIGHT mismatch repair (MMR) genes (Thompson et al., 2014), they have incorporated information from *in silico* analyses, clinical information, family history and functional assessments, although the exact data contributing to the assessment depends on the specific qualities of each gene. In all cases, the problem of validation remains, as there is no single "gold standard" of pathogenicity to critically assess the output of quantified models.

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Amongst the most widely used classification strategies for germline variants are the ACMG/AMP guidelines (Richards et al., 2015). These are generic qualitative rules intended to provide recommendations for classification of variants in any gene involved in a Mendelian disorder, and there are currently efforts to adapt these rules to genes of interest, including *TP53* (ClinGen *TP53* Expert Panel, work in preparation). The existing rules, however, do not currently consider all types of evidence that may be relevant for *TP53* variant classification.

In this paper, we describe a quantitative model to predict pathogenicity of missense variants in *TP53* that is an alternative to the qualitative ACMG/AMP guidelines. The model combines outputs of bioinformatic tools (previously selected as high-performing for *TP53* (Fortuno, James, Young, et al., 2018)), with a novel approach to incorporate reports of variation detected in tumors in comparison to the reported germline frequency - referred to as the somatic to germline ratio (SGR). We apply this quantitative model to every possible p53 missense variant and examine its performance by comparing the model-derived pathogenicity assignments to clinical predictors of variant pathogenicity from a range of independent datasets.

MATERIALS AND METHODS

This research has been approved by the Human Research Ethics Committee of the QIMR Berghofer Medical Research Institute.

Reference sets of assumed pathogenic and assumed benign p53 missense variants

Reference sets of assumed pathogenic and assumed benign missense variants were selected for subsequent analyses, using strict functional and clinical evidence as

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described in Table 1; details of reference set variants have been published previously (Fortuno, James, Young, et al., 2018), and are shown in Table 1.

Variants are described on *TP53* genomic reference sequence NC_000017.10, transcript variant NM_000546.5 and on SwissProt protein sequence #P04637.

Calibration of Align-GVGD

Align-GVGD (http://agvgd.hci.utah.edu) is a gene-specific tool that considers physicochemical properties of amino acids and evolutionary conservation. The Grantham variation (GV) and deviation (GD) scores were calculated for each variant as previously described (Mathe et al., 2006), using an optimized protein multi-sequence alignment (Fortuno, James, Young, et al., 2018). Overall, 76% of the assumed benign variants and 11% of assumed pathogenic variants fell into the lowest A-GVGD category (C0), while 59% of assumed pathogenic variants and none of the assumed benign variants fell in the highest category (C65) (Mathe et al., 2006; Tavtigian et al., 2006). The highest GV (>353) occurs due when a gap is present in the protein multi-sequence alignment inflating the value, and these variants were removed from reference sets for this purpose (any variant with this GV was assumed to have a LR of 1). The likelihood that an individual variant fell into either the pathogenic or benign reference sets was calculated based on both the GV and GD. The standard pathogenic variants showed limited variation in GV (78% had a GV=0) and similarly variation in GD was limited for assumed benign variants. As a result, a parsimonious estimate of pathogenicity can be given by the LR:

$$\mathcal{L}_i = \frac{P(Vp_i|GD)}{P(Vn_i|GV)}$$

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where *P(Vp)* is the probability that the variant *i* is pathogenic according to its GD value and *P(Vn)* the probability that it is benign according to its GV value.

Calibration of BayesDel

BayesDel is an accurate ensemble method which combine multiple tools with different algorithms (Feng, 2017), but does not include Align-GVGD. BayesDel scores were calculated for all variants in the reference sets. Average score for the pathogenic reference set was 0.44, while the benign reference set had an average of -0.01. Following a similar approach as described above for Align-GVGD, an estimate of pathogenicity can be given by the LR:

$$\mathcal{L}_i = \frac{P(Vp_i|BDs)}{P(Vn_i|BDs)}$$

where *P(Vp)* is the probability that the variant *i* is pathogenic according to its BayesDel score value and *P(Vn)* the probability that it is benign according to its BayesDel score.

Somatic to Germline ratio (SGR)

A unique strength of the IARC *TP53* Database (Bouaoun et al., 2016) variants is the depth of data on the frequency with which variants are reported somatically from tumor sequencing as well as in the germline from sequencing individuals with familial cancer. Somatic *TP53* variants are common drivers in many types of cancer. The presence of these variants in the database reflects selection in the tumor for their pathogenic effect, while the comprehensive nature of the data is such that absence of reports from the somatic setting is equally evidence against pathogenicity. However, no clear method to quantify the strength of this evidence has been described.

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When considering variant counts, it is useful to consider variants in different classes. In the case of pathogenic variants in the germline, we can consider that these are being removed from the population at a certain rate, due to their effect on fitness, and at the same rate are reintroduced as *de novo* mutations. Consequently, pathogenic variants in both the germline and somatic samples should occur at a rate that in part reflects the nucleotide specific mutation rate. In contrast, for the class of benign variants, there is no similar relationship between the frequency at which they become fixed in the population and somatic counts of the same variant, which if they are observed occur essentially by chance in a tumor. There is also a third class of variants reported somatically at high frequency due to the specific effect of a known environmental mutagen, such as aflatoxin (p.Arg249Ser) (Olivier, Hollstein, & Hainaut, 2010). Twelve variants with these properties are described (p.Val157Phe, p.Arg158Leu, p.Arg273Leu, p.Asn131Ile, p.Asn131Tyr, p.Arg209Ser, p.Arg280Ser, p.Arg249Ser, p.Arg249Met, p.Arg248Trp, p.Arg249Ser, p.Arg249Met). Such variants have very rarely or never been reported as germline variants, and were removed from the pathogenic reference set for the purpose of deriving a likelihood for predicting pathogenicity of germline sequence variants. The p.Pro72Arg and p.Pro47Ser variants were also removed from the benign reference set for this approach, as they are known “polymorphisms” and therefore have an extremely high number of germline counts in comparison with other assumed benign variants, and somatic counts were excluded from the IARC *TP53* Database. Similarly, the variant p.Arg337His was also excluded since germline frequencies are affected by a founder effect in Brazilian populations.

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To quantify the evidence from somatic variant counts to indicate pathogenicity of a variant in the germline, we began by examining the relationship between somatic (IARC *TP53* Database (R19, August 2018)) and germline counts (IARC *TP53* Database, ExAC-nonTCGA) by linear regression. This confirmed the strong positive correlation for pathogenic reference sets (R^2=0.739), and no similar correlation between the benign reference sets (R^2=0.012) (Figure 1). As reference sets were selected using clinical and population information, that potentially correlates with the reported counts, we repeated the analysis for variants selected based on transactivation activity data only and observed the same trend (R^2=0.823 for non-functional variants versus R^2=0.027 for functional variants).

As a consequence, this creates an expectation that the number of somatic counts will differ for pathogenic versus benign variants. Moreover, the distribution of the regression residuals for the pathogenic and benign variant correlations can be used to determine the probability that the observed value falls within either of the two expected distributions. The calculation was performed for the distribution of the natural log of the regression residuals to reduce the magnitude of the resulting LR to a level that is meaningful in clinical practice (see Supplementary Methods). The final LR is expressed as:

$$\mathcal{L}_i = \frac{P(Vp_i|S,G)}{P(Vn_i|S,G)}$$

where *P(Vp)* is the probability that the variant *i* is pathogenic according to its number of somatic and germline counts and *P(Vn)* the probability that it is benign according to its number of somatic and germline counts.

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This LR has the expected characteristic in that, for rare variants with few counts in either the germline or somatic setting, the value remains very close to 1. For variants with an increasing number of germline counts, if somatic counts of the same variant also increase in line with the expectation of a pathogenic variant, then the LR increases correspondingly. Conversely, for variants that are reported frequently in the germline without significant numbers of somatic counts, the inverse of the LR rises, giving stronger evidence against pathogenicity. For variants that have not been reported in the germline to date, the analysis calculated the LR for a putative future occurrence. For variants with no somatic counts in tumors, or only one somatic count and no germline counts, we considered there is not enough evidence to apply this value in quantitative modelling and therefore these variants could not be classified yet.

Application of the quantitative model to all missense variants

After proving that there was no linear correlation among the three components of the model (R^2 AlignGVGD vs BayesDel = 0.16, R^2 SGR vs Align-GVGD = 0.0003, R^2 SGR vs BayesDel = 3E-05), these components were combined to provide an assessment of pathogenicity that incorporates the independent data relating to changes in amino acid physicochemical properties, evolutionary conservation, and previous counts of germline and somatic events – where the combined LR is the product of the individual LRs. LRs from bioinformatic tools were available for all possible 2314 missense variants (original outputs can be found in Supp. Table S4 of Fortuno et al., 2018), while LRs from SGR were available for 1121 single nucleotide substitutions (1007 unique missense variants) with enough germline/somatic counts. A posterior probability of pathogenicity could therefore be calculated for 1121 missense variants. A final classification of

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variants based on posterior probability of pathogenicity was made following the IARC five class scheme as previously described (Plon et al., 2008): pathogenic (P, with posterior >0.99), likely pathogenic (LP, 0.95-0.99), variant of uncertain significance (VUS, 0.05-0.949), likely benign (LB, 0.001-0.049), and benign (B, <0.001).

Datasets used for validation of the quantitative model

A number of datasets were explored to examine the validity of the quantitative model described.

Differences in age of diagnosis by variant class

Early onset of cancers is a striking feature for carriers of *TP53* pathogenic variants compared to the general population (Olivier et al., 2003). The IARC *TP53* Database contains information on the age of diagnosis for a large number of index cases. Clinical data was obtained from confirmed carriers of missense variants, and variants classifications were made following the quantitative model. The Student's t-test with normalized ages was used to find significant differences between pathogenic variant carriers versus benign variant carriers.

Classification of variants in different clinical subgroups

Variants documented from specific groups of patients or healthy individuals are expected to be enriched or depleted for pathogenic variants, respectively. Two specific groups of variants were compared, and classified by our quantitative model: i) variants reported in the IARC *TP53* database in individuals meeting Classic LFS criteria, a variant group that would be expected to enriched for pathogenic variants, and ii) variants in women over 70 years without a personal history of cancer from the Fabulous Ladies Over Seventy database (FLOSSIES, https://whi.color.com/), where true pathogenic variants would be expected to be very rare, although a higher frequency of clonal hematopoiesis is anticipated in this aged population. In

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addition, we also calculated the proportion of pediatric probands (diagnosed before 18 years of age) carrying missense variants classified by our quantitative model as pathogenic or likely pathogenic, versus benign or likely benign.

Correlation with p53 function measured using yeast transactivation assays

As noted, the transactivation activity of every possible p53 missense variant has been analyzed in yeast assays (Kato et al., 2003). Although transactivation activity does not equal pathogenicity, we expected non-functional variants to be enriched for pathogenic variants, and functional variants to be enriched for benign variants. We therefore compared the proportion of variants with different ranges of transactivation activity in variants classified by our model. As this type of evidence was also used to select variants in our reference sets for model component assessment and calibration, reference set variants were excluded for this validation.

Correlation with loss of p53 function measured in human cells

The outputs of the model were also compared to three datasets from human cellular models. In a recent study (Kotler et al., 2018), a massively parallel mutational scan was used to measure the functional impact of ~10,000 *TP53* variants in the DNA Binding Domain (DBD) in vitro, deriving a relative fitness score for each variant (RFS). High RFS scores corresponded to variants that strongly disrupted wildtype-p53 anti-proliferative functionality whereas lower RFS scores represented variants that retained anti-proliferative function. A cut-off of -1 was reported to give the best separation between both groups of variants. We compared the outputs of our quantitative model with the reported RFS.

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Giacomelli et al., 2018 also performed a recent systematic analysis of almost all possible single nucleotide variants in the full length *TP53* sequence, using different conditions in a human cell model to independently assess the fitness advantage conferred by loss-of-function (LOF). Outcomes were reported as the relative enrichment (Z score) of individual variants under the selective conditions, which again allowed comparison to the output of the model. In a conservative approach to infer LOF, the cut-off selected was -0.21 for etoposide assays, which showed the best separation between silent and common cancer missense variants as reported in the published data (Figure 2F in Giacomelli et al., 2018).

Finally, we used a clonogenic assay to examine the twenty two *TP53* variants (17 missense, 2 nonsense, 1 frameshift, 2 in-frame indels) previously identified in 23 unrelated sarcoma probands from the International Sarcoma Kindred Study (ISKS) cohort (Ballinger et al., 2016). This analysis employed the p53-null human H1299 cell line (Mitsudomi et al., 1992) derived from a non-small cell lung carcinoma. The pCMV-Neo-Bam-p53 plasmid (Lin, Chen, Elenbaas, & Levine, 1994) containing wild-type human p53 cDNA was utilized to generate the mutant variants by site directed mutagenesis and the p53 mutant constructs used to transfect the H1299 cells in triplicate. The cells were split after 24 hours and subjected to a colony-formation assay involving an automated count of colonies containing at least 50 cells. Controls were represented by the empty vector, the vector carrying the wild-type form of *TP53* and two LOF *TP53* mutants, c.524G>A; p.Arg175His and c.818G>A; p.Arg273His (Dittmer et al., 1993). Detailed information about these assays can be found in Supplementary Methods. The functional properties observed for the missense variants in these assays were

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compared with the outputs of the quantitative model for the same variants, when classifications were available.

Comparison to existing classifications submitted to the ClinVar database

The ClinVar database collates and presents data on the classification of variants based on the elective contributions of testing laboratories that submit details of their variant curation (Landrum et al., 2018). The database includes entries for 596 missense variants (accessed September 10, 2018). We compared the 'Clinical significance" categories recorded in ClinVar for these 596 variants with the classification outputs of our quantitative model.

RESULTS

Quantitative analysis of missense variants in *TP53*

The calibrated Align-GVGD analysis discriminated between the reference sets of assumed pathogenic and benign variants: median LR 2.54 (range 0.29-3.95) versus 0.99 (range 0.29-3.88). After assessing all 2314 missense variants, it was observed that the average LR increased with the increasing Align-GVGD category from C0 to C65. The continuous quantified value provided considerable further discrimination within each category (Figure 2).

Discrimination between reference set LRs was stronger for BayesDel, with a median LR of 38.63 (range 0-122) for assumed pathogenic variants and 0.06 (range 0-69) for assumed benign variants, as observed in Figure 3A. Finally, SGR also strongly discriminated between pathogenic variants (median 8.94; range 0.07 - >1,000) and benign variants (median 0.07; range 0-10.3) (Figure 3B).

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Combining these three components, sufficient data was present to allow classification of 1121 missense variants in *TP53* (Table 2). All resulting LRs from each component in the quantitative model for each possible missense variant can be found in Supp. Table S1.

Validation of the quantitative model outputs

Several approaches were taken to validate the classifications generated by application of the model. A summary of the validation results is shown in Table 3 and detailed results of each approach are provided in the next subsections.

Clinical information correlates with variant pathogenicity assigned using the quantitative model

The average age of cancer diagnosis in *TP53* germline pathogenic variant carriers reported in the IARC *TP53* database was compared between carriers of P or LP (P/LP) (n=422) versus B or LB (B/LB) (n=36) variants, following classification by our quantitative model. Average age of diagnosis was significantly lower for P/LP carriers than for B/LB carriers (24.7 vs 35.8; p-value = 0.0004). In addition, of 253 probands with missense variants in the IARC *TP53* database diagnosed with a pediatric malignancy, 157 (62.0%) carried a variant classified as P/LP versus only 4 (1.6%) who carried a variant classified as B/LB. Further, all of the variants reported in confirmed *de novo* probands with no family history (9/9), and 38/39 variants in Classic LFS probands (total 97.9%), where a quantitative classification other than VUS was assigned, were classified as P/LP. The discordant variant was p.Asn235Ser, with functional transactivation class and 55 alleles in gnomAD. In contrast, 77.8% (7/9) of the variants found in women from FLOSSIES (cancer-free, aged over 70 years) with quantitative classifications available other than VUS were assigned to a B/LB class, and two to LP (both

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reported as "P" in ClinVar). The latter may represent clonal hematopoiesis of indeterminate potential (Spira et al., 2017; Weitzel et al., 2017), but further studies would be needed to assess this possibility.

Results from functional assays correlate with assigned variant pathogenicity

Results from systematic transactivation assays performed in yeast (Kato et al., 2003) were used for comparison with the classifications from our quantitative model (excluding variants used in the original reference sets). Of 236 variants classified as P/LP, 129 (54.6%) had ≤20% activity, another 80 (34.0%) had >20%-<75% activity (average activity of 41.8%), and only 27 (11.4%) had ≥75% activity. Correspondingly, of 272 variants classified as B/LB, 225 (82.7%) had ≥75% activity, another 44 (16.2%) had >20%-<75% activity (average activity of 55.8%), and only three were non-functional (1.1%).

When compared to data from DBD region variants assayed for anti-proliferative function using a high through-put mammalian assay (Kotler et al., 2018), disrupted function was observed for a high proportion of DBD variants classified as P/LP (333/434, 76.7%), and retained function was observed for 159/165 (96.4%) DBD variants classified as B/LB by the quantitative model. When compared to other systematic screen data in human cells (Giacomelli et al., 2018), 311/418 (74.4%) of unique variants classified as P/LP exhibited LOF, as opposed to only 27/244 (11.1%) variants classified as B/LB.

In addition, we compared our results to data from mammalian clonogenic survival assays conducted as part of this study, which also included reduced penetrance p.Arg337His variant as a partial activity pathogenic control. As shown in Figure 4, missense variants within the DBD generally resulted in more severe abrogation

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of protein function, compared to missense variants affecting the NH_2 and COOH terminal domains (transactivation and tetramerization domains, respectively), although five missense variants affecting the DBD displayed activity comparable to the wild type form of the protein. Of the 15 DBD variants also assayed by Kotler et al., 2018, functional results were completely consistent in direction (5 retaining function, 10 disrupting function). The correlation between the performance of the clonogenic assay and systematic transactivation assays (Kato et al., 2003) was moderate, with an R^2 of 0.58. Classification assignment using the quantitative model was available for 17 variants assayed for function using the clonogenic assay: clearly disrupted function was observed for the majority of variants classified as P/LP (10/11; 90.9%), and activity similar to wildtype was observed for the two variants assayed that were classified as LB.

ClinVar "clinical significance" categories are in agreement with assigned variant pathogenicity

Comparison between the model classifications and "clinical significance" categories provided in the ClinVar database is summarized in Figure 5 and detailed in Supp. Table S1. A total of 415 (69.6%) of the 596 variants described in ClinVar were able to be classified by the quantitative model. Overall, out of 124 variants recorded as "P/LP" in ClinVar (without conflicting classifications), our model gave classifications in the same direction for 108 (87.1%), while only one (0.8%) was classified as LB by the model (c.105G>T, p.Leu35Phe). This unique disagreement is based on a somatic event from a paper published in 1993. This specific variant is not present in the germline of any individual in the IARC *TP53* Database and has functional transactivation activity in yeast assays. Similarly, out

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of four variants recorded as "B/LB" in ClinVar, our model classified all of them as LB.

Quantitative model results for variants listed in ClinVar as "VUS" or with "Conflicting interpretations of pathogenicity"

For 230 variants recorded as "VUS" in ClinVar (where quantitative classifications were possible), our quantitative model classified 100 as P/LP, and 61 as B/LB. For a further 57 variants with "Conflicting interpretations of pathogenicity" in ClinVar (with quantitative classifications), 33 were classified as P/LP by our model, and 13 as B/LB. For these variants considered as having "conflicting" ClinVar submissions, the majority of the submissions were in agreement with the classifications made by our quantitative model. Specifically, of the 33 variants assigned a P/LP classification by the model with conflicting submissions in ClinVar, all of them had at least one submission as "P/LP" (total 259), and 1-2 "VUS" submissions each, but none had a submission as "B/LB". Finally, of the 13 B/LB classified variants with conflicting submissions in ClinVar, all but one had at least one "LB" submission (total 21) and 1-4 "VUS" submissions each; the single exception was c.91G>A, p.Val31Ile, with one "B", three "LB", two "VUS" and one "LP" submission.

We detail in Table 4 the list of 24 variants (absent from reference sets) currently recorded in ClinVar as "VUS" or with "Conflicting interpretations of pathogenicity", to which our model assigned a posterior probability of pathogenicity ≥ 0.999.

DISCUSSION

Germline variants that abrogate the tumor suppressor function of the *TP53* gene

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result in possibly the highest lifetime risks of cancer for any individual risk factor. The breadth of associated cancers, the frequency of *de novo* mutations, strengthening evidence of effective screening and risk reducing strategies (Ballinger et al., 2017; Villani et al., 2016), and the rapidly falling cost of multigene panels, all suggest an increasing role for sequencing of *TP53* in clinical practice. The majority of *TP53* sequence variants are predicted to encode missense changes, which are difficult to interpret clinically. Variant classification models that incorporate multiple data types, where each component is statistically weighted to reflect the strength of evidence for (or against) pathogenicity have been used successfully for variant interpretation of several different cancer syndrome genes (Spurdle et al., 2012; Spurdle et al., 2008; Thompson et al., 2014), and here we have developed the first quantitative model for *TP53* variant classification. We estimated probability of pathogenicity using two bioinformatic tools previously selected as the best-performing tools for *TP53* (Fortuno, James, Young, et al., 2018), but in this analysis we converted the binary outputs to a continuous range of LRs for improved discrimination between variants. In addition, we used the relationship between somatic and germline counts (SGR), assisted by the collection of data available through the IARC *TP53* Database (Bouaoun et al., 2016), as a measure of the effect of genetic variation on *TP53* gene function. The model derives new clinical interpretations for many variants currently considered VUS, and provides a foundation for a wider quantified approach as further lines of evidence such as familial segregation and tumor pathology are able to be incorporated as statistically quantified components.

As there is no ‘gold standard’ to determine pathogenicity of variants, we compared the performance of the quantitative model with a number of clinical

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predictors, functional data, and existing ClinVar classifications and demonstrated a good correlation overall. Variants classified as pathogenic or likely pathogenic by the model were associated with earlier age of cancer onset (including a greater proportion of pediatric cancers), and were enriched in Classic LFS probands and depleted in a cohort of cancer-free women over 70 years. When compared to the output of reported functional assays, the majority of variants classified as pathogenic or likely pathogenic were non-functional in transactivation yeast assays (Kato et al., 2003), showed disruption of anti-proliferative function/LOF (Giacomelli et al., 2018; Kotler et al., 2018), or reduced activity in the mammalian clonogenic survival assays developed in this study. Variants classified as benign or likely benign variants showed the same trend in the opposite direction. Of note, the three variants classified as pathogenic by our model that showed activity comparable to wildtype p53 using clonogenic assays are all reported as "VUS" in ClinVar, and two have partial yeast transactivation activity. Finally, classifications from the quantitative model were in agreement with ClinVar reports for both pathogenic and benign variants.

A number of limitations and assumptions can be identified in the quantitative model presented. Although care has been taken to avoid the repeated use of any component of the data in generating the LRs, there is an assumption that each element provides a truly independent assessment of pathogenicity. The tools assume a naïve prior probability for the pathogenicity of any missense variant of 0.5 (i.e. favoring neither pathogenic nor benign assignment). This is similar to the published model for MMR genes (Thompson et al., 2014), but higher than for *BRCA1/BRCA2* (Tavtigian, Byrnes, Goldgar, & Thomas, 2008). In the case of *TP53*, where missense variants represent the largest group of pathogenic variants,

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this prior risk may even be considered conservative. Finally, we only applied this model to variants with at least one somatic and more than one germline count. Of the variants excluded for this reason, some may have no somatic counts and a high number of germline counts. If we were going to apply the SGR approach for such variants applying a putative somatic occurrence, the resulting LR would be against pathogenicity. These calculations should be revisited in the future when there are more somatic data available or additional pieces of evidence in the quantitative model. Development of our quantitative model also highlighted a number of issues. In the analysis of the reported somatic occurrences, specific known 'hotspots' are commonly the result of specific environmental mutagens (Olivier et al., 2010), but germline variants are rare or have never been reported. For this small number of environmentally-driven somatic hotspots, the high number of somatic counts has the potential to produce an over-estimate of the LR for the same variant in the germline. A more general issue for a quantified model is how to clinically interpret variants assigned the classification of 'uncertain significance' after considering all available evidence. A variant may be assigned to this class reflecting the limited information available to generate a classification, and/or because the model, as described, is derived using predictors of pathogenicity for classical "high-risk" variants and is not capable of accurately assessing the effects of variants associated with reduced penetrance. Incorporation of additional information into future iterations of the model, in particular the growing data from functional assays, calibrated against clinical measures of risk, may prove useful to identify variants in this category.

We previously proposed the use of BayesDel and Align-GVGD in the context of the ACMG/AMP computational rules (PP3, PP3_Moderate, and BP4) for *TP53*

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(Fortuno, James, Young, et al., 2018). The other component of the model (SGR), however, is not fully considered by any ACMG/AMP rule. Given the increasing popularity of these guidelines and the efforts to adapt them to genes of interest, we propose the conversion of the SGR component to a new rule for users of the ACMG/AMP guidelines, in line with the recent Bayesian re-analysis of the ACMG/AMP framework (Tavtigian et al., 2018). That is, the LR output from the SGR could be equated to an ACMG/AMP rule of varying strength depending on its value, which would need a new code. Based on the LRs resulting from SGR analysis, 49 missense variants would meet the ACMG category “Very Strong” for pathogenicity, 95 would meet the category “Strong”, 130 would meet the category “Moderate”, and 105 would meet the category “Supporting” for pathogenicity. Similarly, 15 missense variants would meet the ACMG category “Strong” of benign impact and 387 would meet the category “Supporting” of benign impact (Supp. Table S1). This proposed new rule should not be used in combination with ACMG/AMP PM1 (variant located in a mutational hotspot and/or critical and well-established functional domain without variation). For *TP53*, the PM1 code could be applied for missense variants occurring at major hotspot amino acid residues (175, 245, 248, 249, 273, 282) and potentially other recurrently implicated codons that are yet to be defined (Chang et al., 2018; Walsh et al., 2018). The proposed SGR rule has the advantage of providing a single approach that enables prediction of pathogenicity for both hotspot and non-hotspot missense variants.

In summary, we have developed a novel quantitative model to classify missense variants in *TP53*, by applying outputs from high-performing bioinformatic tools transformed into LRs and integrating a feature specific to *TP53*; the relationship

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between somatic and germline counts. These initial approaches could be further augmented by the incorporation of additional quantitative evidence to the model, derived from other data sources such as the number of other pathogenic variants at a specific residue, independent functional data (calibrated against clinical information), personal and family history analysis, and/or information on the histo-pathology of tumors.

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CONFLICT OF INTEREST

KT has chaired 2 meetings and received honorarium for lectures from AstraZeneca.

Remaining authors declare no conflicts.

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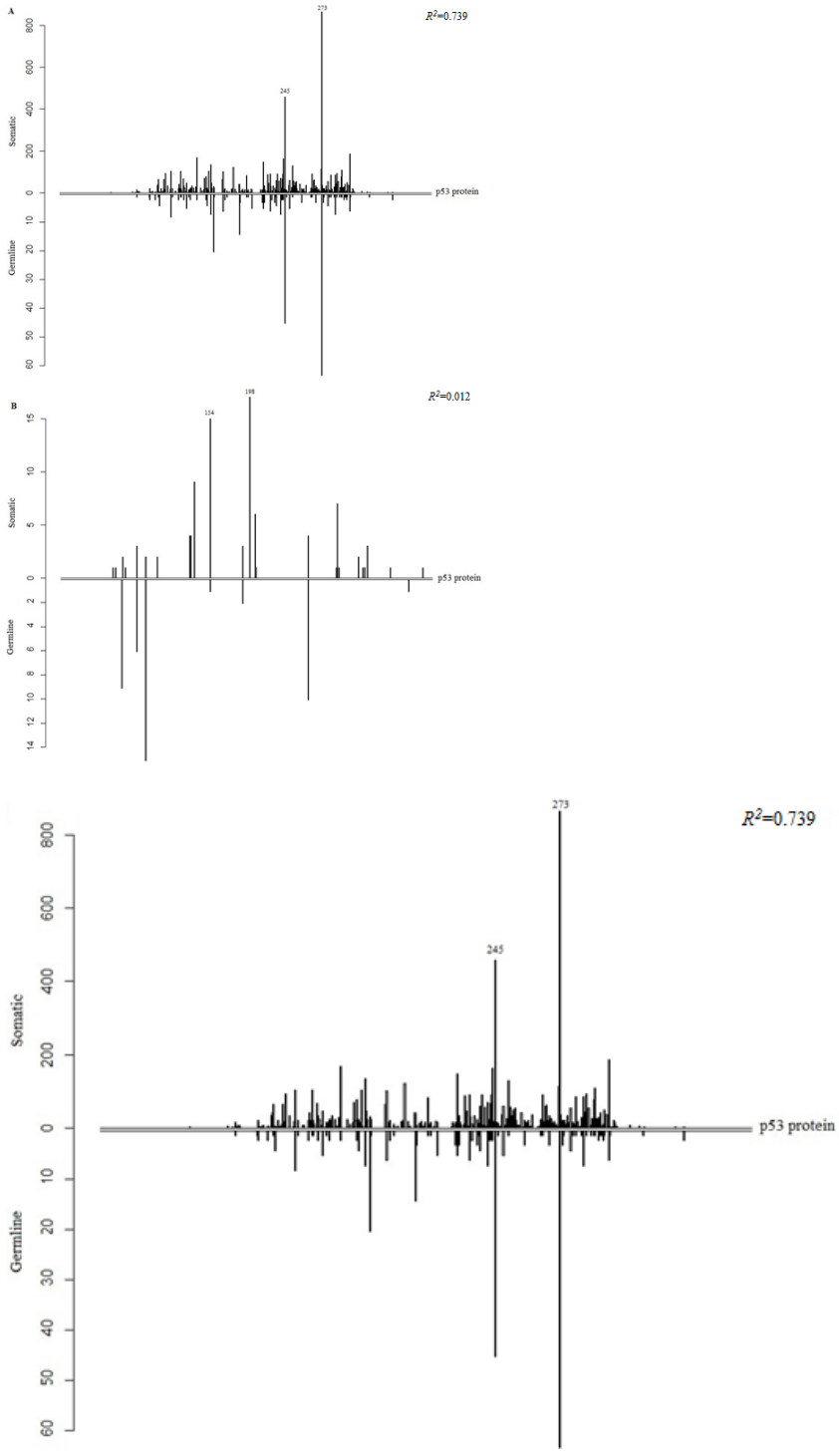
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FIGURES

Figure 1. Relationship between the number of somatic (above the line) and germline (below the line) counts in the IARC *TP53* Database (Bouaoun et al., 2016) by protein position for assumed pathogenic (A) and benign (B) variants used to estimate the SGR likelihood ratio (see text for description of the selection of variants).



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Figure 2. Distribution of LRs for all possible 2314 p53 missense variants in *TP53* according to their Align-GVGD Class

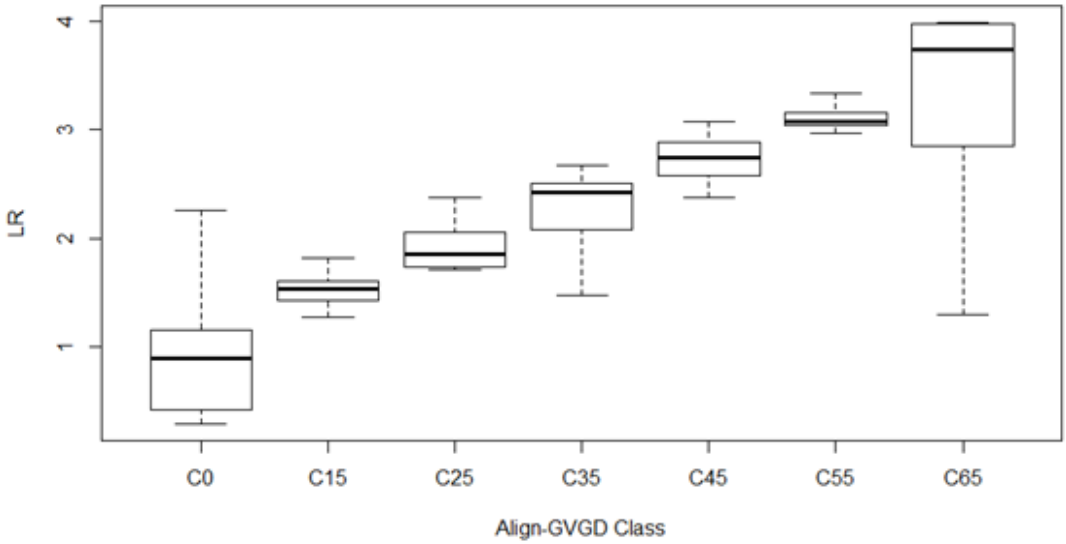
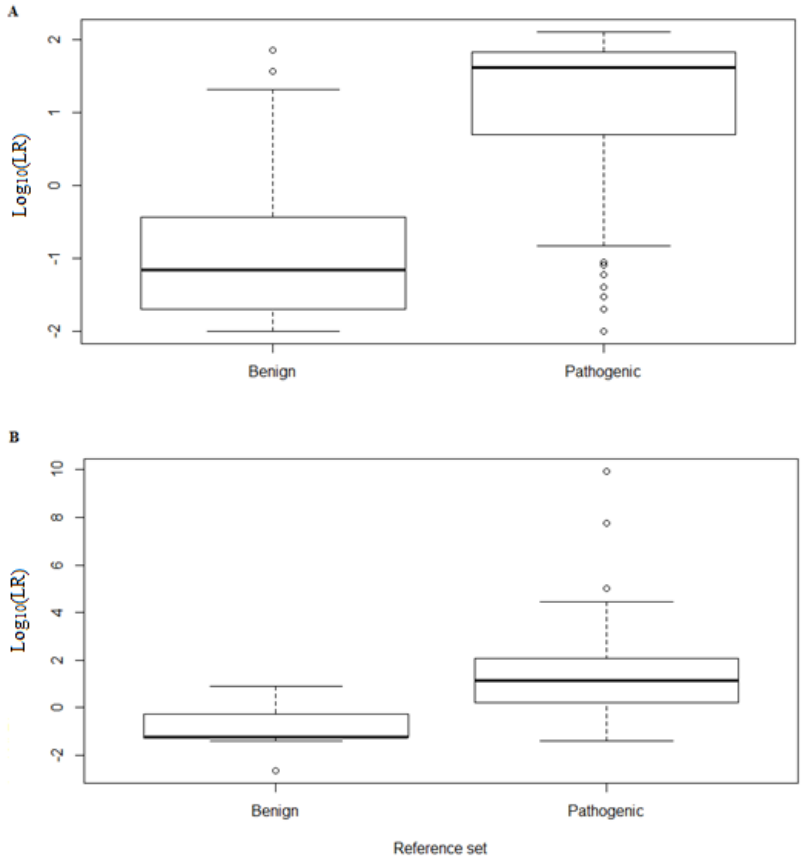


Figure 3. Distribution of BayesDel (A) SGR (B) LRs (Log10) in variants from assumed benign and assumed pathogenic reference sets



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Figure 4. Characterization of the functional impact of sarcoma-associated germline *TP53* variants in human cells by clonogenic assays. (A) The ability of *TP53* variants to induce apoptosis in human cancer cells was evaluated by clonogenic assays in p53-null H1299 cells transiently transfected with a plasmid carrying different variants. Data have been normalized and are expressed as percentage of colony suppression vs the wild-type form of the protein. The functional impact of three well characterized pathogenic missense variants is highlighted by the dashed line for p.Arg175His, p.Arg273His and the solid line for the reduced penetrance Brazilian founder variant p.Arg337His (associated with partial function in yeast transactivation assays). For each variant, transfections were carried out as multiple replications in repeated independent experiments (each represented as a dot). Black symbols $p \geq 0.05$, grey symbols $p < 0.05$. Seven variants demonstrated activity comparable to (or greater than) the range of activity observed for wildtype protein. All other variants assayed demonstrated significantly less activity than the wildtype control, although the range of activity observed for the p.Arg333Cys variant suggests that this variant may represent an intermediate activity allele. (B) Schematic representation of the p53 mutants within the structural domains of the protein. Numbers indicate amino-acid residues.

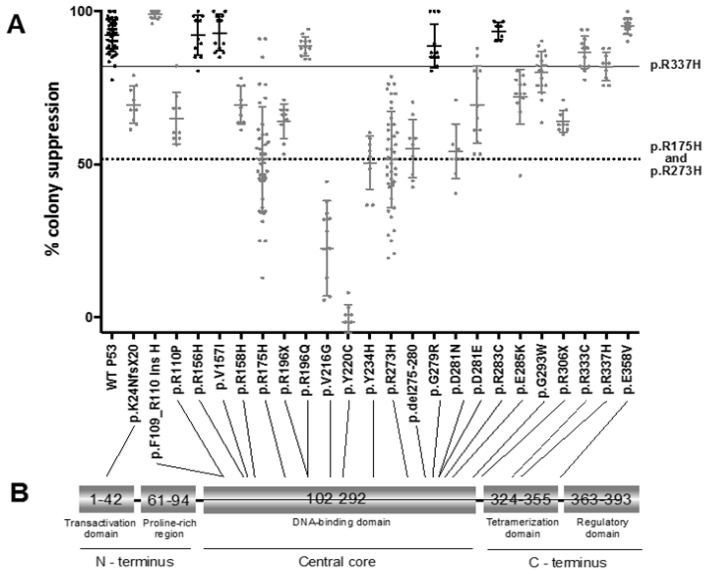
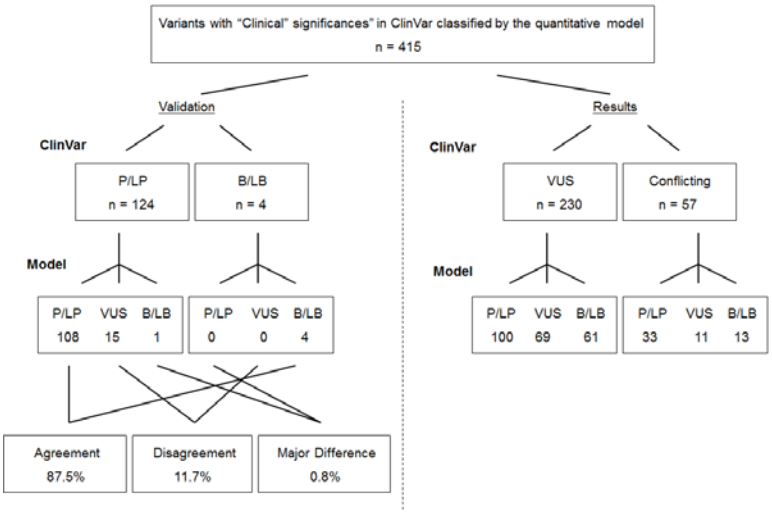


Figure 5. Comparison of ClinVar "Clinical significance" categories with quantitatitative model results for p53 missense variants. P = Pathogenic, LP = Likely Pathogenic, VUS = Variant of Uncertain Significance, LB = Likely Benign, B = Benign.



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Table 1. Definition of reference sets of assumed pathogenic and assumed benign missense variants in *TP53*

Reference sets	
Assumed pathogenic (n=247)	**Assumed benign (n=69)***
Variants with ≤20% activity in each transactivation assay (Kato et al., 2003) OR Variants found in tumors from the cBioPortal database (Cerami et al., 2012) with reported dominant-negative effect according to the IARC *TP53* Database (Bouaoun et al., 2016) AND absent in ExAC-nonTCGA/gnomAD population databases (Lek et al., 2016)	Variants with ≥75% - ≤140% activity in each transactivation assay (Kato et al., 2003) OR Variants with ≥0.03% allele frequency in at least one population in ExAC-nonTCGA/gnomAD population databases (excluding Ashkenazi Jewish) (Lek et al., 2016) AND absent in IARC *TP53* Germline database (Bouaoun et al., 2016)

*Two missense variants that were included in the assumed benign reference set by Fortuno et al., 2018 (p.Glu339Lys and p.Gly360Ala) have now been excluded since they are now present as germline variants in the latest release of the IARC *TP53* Database (R19, August 2018).

Table 2. Assigned variant pathogenicity of missense variants in *TP53* using our quantitative model

Classification	**N (%)**
Pathogenic (P)	314 (28.0%)

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Likely pathogenic (LP)	127 (11.3%)
Variant of uncertain significance (VUS)	391 (34.9%)
Likely benign (LB)	259 (23.1%)
Benign (B)	30 (2.7%)

Table 3. Summary of clinical, functional, and ClinVar validation results for variants classified as pathogenic or likely pathogenic (P/LP) or benign or likely benign (B/LB) using our quantitative model

Validation		**P/LP classified variants**	**B/LB classified variants**
Clinical	**Average age of cancer diagnosis**	24.7y	35.8y
	Pediatric probands	62%	1.6%
	Classic LFS/de novo probands	97.9%	2.1%
	FLOSSIES women	22.2%	77.8%
Functional	**Transactivation assays (Kato et al., 2003)**	54.6% ≤20% activity	1.1% ≤20% activity
	Anti-proliferative function (Kotler et al., 2018)	76.7% disruptive function	3.6% disruptive function
	LOF (Giacomelli et al., 2018)	74.4% LOF	11.1% LOF
	Clonogenic survival assays	90.9% disrupted function	0% disrupted function

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ClinVar	87.1% “P” or “LP”	0% “P” or “LP”

Table 4. Variants strongly classified as pathogenic by our quantitative model recorded as having "Uncertain significance" (VUS) or "Conflicting interpretations of pathogenicity" (Conflicting) in ClinVar (data from September 2018)

Transcript Change	Protein Change	ClinVar Clinical Significance	Combined LR
c.817C>T	p.Arg273Cys	Conflicting	744587
c.536A>G	p.His179Arg	VUS	521313
c.581T>G	p.Leu194Arg	VUS	93792
c.711G>A	p.Met237Ile	Conflicting	25321
c.413C>T	p.Ala138Val	VUS	17017
c.811G>A	p.Glu271Lys	VUS	13426
c.830G>T	p.Cys277Phe	VUS	6188
c.641A>G	p.His214Arg	Conflicting	5375
c.380C>T	p.Ser127Phe	Conflicting	4607
c.526T>A	p.Cys176Ser	VUS	3574
c.490A>G	p.Lys164Glu	VUS	3184
c.394A>G	p.Lys132Glu	Conflicting	3087
c.785G>T	p.Gly262Val	VUS	2332
c.434T>C	p.Leu145Pro	VUS	1741

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c.388C>G	p.Leu130Val	VUS	1730
c.724T>A	p.Cys242Ser	VUS	1553
c.764T>C	p.Ile255Thr	VUS	1467
c.523C>T	p.Arg175Cys	VUS	1361
c.746G>A	p.Arg249Lys	VUS	1330
c.569C>T	p.Pro190Leu	VUS	1312
c.845G>A	p.Arg282Gln	Conflicting	1310
c.832C>G	p.Pro278Ala	VUS	1225
c.454C>T	p.Pro152Ser	VUS	1112
c.530C>T	p.Pro177Leu	VUS	1003

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