Benchmarking *in silico* Tools for the Functional Assessment of DNA Variants using a Set of Strictly Pharmacogenetic Variants
(Ujian Tanda Aras Alat *in silico* untuk Penilaian Kesan Fungsian Varian DNA menggunakan Varian Farmakogenetik Terpilih)

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**ABSTRACT**

Predictive algorithms are important tools for translating genomic data into meaningful functional annotations. In this work, we benchmarked the performance of eight prediction methods using a set of strictly pharmacogenetic variants. We first compiled a set of damaging or neutral variants that affected pharmacogenes from two online databases. We then cross-checked their functional impacts against the predictions given by the chosen tools. Of the eight methods, SIFT (*Sorting Intolerant From Tolerant*), Mutation Assessor, and CADD (*Combined Annotation Dependent Depletion*) were the top performers in predicting the functional relevance of a variant. The performance of SIFT surpassed that of CADD despite its much simpler algorithm, correctly identifying 66.91% of the damaging variants and 84.38% of the neutral variants. SIFT assumes that important DNA bases within a gene are conserved and not amenable to substitution. Overall, none of the prediction methods struck a balance between sensitivity and specificity. For instance, we noted that CADD was very sensitive in detecting the damaging variants (89.21%); however, it also mispredicted a large fraction of the neutral variants (43.75%). We then trialled a consensus approach whereby the functional significance of a variant is defined by agreement between at least three prediction methods. The approach performed better than all the tools deployed alone, detecting 84.17% of the deleterious variants and 70.97% of the neutral variants. A prediction method that integrates an assortment of algorithms, each assigned an empirically optimised weighting, may be established in the future for the functional assessment of pharmacogenetic variants.

**Keywords:** Deleteriousness; functional impact; pharmacogenetic variant; predictive algorithm; receiver operating characteristic analysis

**ABSTRAK**

Algoritma ramalan merupakan alat yang penting dalam menterjemahkan data genom kepada anotasi fungsian yang lebih bermakna. Dalam kajian ini, kami menilai prestasi lapan kaedah ramalan dalam mengenal pasti kesan fungsian varian farmakogenetik. Kami mengumpulkan varian farmakogenetik yang neutral atau merosakkan fungsi protein daripada dua pangkalan data atas talian. Kemudian, kami membandingkan kesan fungsian setiap varian tersebut dengan ramalan yang dijanakan oleh kaedah yang terpilih. Daripada lapan kaedah tersebut, SIFT (*Sorting Intolerant From Tolerant*), atau Mengasingkan Varian Mudarat Daripada Varian Neutral), Mutation Assessor dan CADD (*Combined Annotation Dependent Depletion*, atau Kesusutan Bersandarkan Anotasi Gabungan) adalah terbaik dalam menentukan kesan fungsian sesuatu varian farmakogenetik. SIFT mencapai prestasi yang lebih baik daripada CADD walaupun algoritmanya lebih ringkas. Kaedah tersebut dapat mengenal pasti 66.91% varian yang mencacatkan fungsi protein dan 84.38% varian neutral. Ramalan SIFT adalah berasaskan anggapan bahawa bes DNA yang penting dalam sesuatu gen adalah terabadi dan tidak boleh ditukar ganti. Secara keseluruhan, tiada kaedah ramalan mencapai keseimbangan antara peka dan kekhususan diagnostik. Contohnya, kami mendapati bahawa CADD sangat sensitif dalam mengesankan varian mudarat (89.21%); tetapi, CADD juga membuat ramalan yang salah terhadap kesan fungsian sekelompok besar varian neutral (43.75%). Seterusnya, kami menaksirkan kesan fungsian varian dengan berdasarkan persetujuan antara sekurang-kurangnya tiga kaedah ramalan. Pendekatan konsensus ini didapat lebih baik daripada menggunakan mana-mana kaedah secara bersasingan. Pendekatan tersebut mampu mengesankan 84.17% varian mudarat dan 70.97% varian neutral. Kaedah ramalan yang menggabungkan pelbagai algoritma, dengan setiap satunya diberi pemberatan yang ditentukan secara empirik, mungkin dibangunkan pada masa hadapan bagi menilai impak fungsian varian farmakogenetik dengan lebih berkesan.

Kata kunci: Algoritma ramalan; analisis penerimaan pengoperasian lengkung; impak fungsian; mudarat; varian farmakogenetik
INTRODUCTION

In silico algorithms for estimating the functional effects of DNA variants render genomic data meaningful. The basis for pinpointing a functionally significant variant is fairly intuitive: Gene regions encoding key biological functions are conserved and variation in these segments is non-permissible and therefore detrimental. This constitutes a chief assumption at the core of many predictive algorithms (Adzhubei et al. 2010; Kircher et al. 2014; Ng & Henikoff 2003; Reva et al. 2011; Tang & Thomas 2016). However, the assumption may fall short of capturing variants that impair less crucial processes such as drug disposition. For instance, individuals deficient in CYPs (cytochromes P450) lead entirely normal lives apart from the occasional distress when they are given medications dependent on the enzymes for elimination. This has allowed an assemblage of quiescent CYP variants to persist in the general population at relatively high frequencies, in contrast to their more penetrant, disease-causing counterparts (Gardiner & Begg 2006).

Hence, we speculated that in silico algorithms tailored for the assessment of pathogenicity might be unsuitable for pharmacogenetics variants. For instance, in our preliminary analysis of a well-known prediction tool, Combined Annotation Dependent Depletion or CADD, we noted that the tool gave an unacceptably large number of false-positives. CADD is a relatively novel member of the growing family of predictive algorithms. Owing to its purported versatility, being effective against both coding and non-coding DNA variants, CADD is steadily gaining popularity in pharmacogenetic research (Bush et al. 2016). However, one notable caveat of CADD is that it gives much weight to the influence of purifying selection when assessing the functional relevance of a DNA variant, which may be weak against pharmacogenetic variants and thus somewhat irrelevant (Li et al. 2014).

Further, pharmacogenetic variants have been shown to induce various molecular changes that could be hard to detect; in some instances, they alter enzyme activity in a substrate-specific manner (Sakuyama et al. 2008; Yu et al. 2002). In this work, we evaluated the performance of eight prediction methods with distinct mechanisms of gauging the functional relevance of a DNA variant, which we theorised that a combination of several methods would outperform individual algorithms used alone in predicting the functional effects of pharmacogenetic variants.

MATERIALS AND METHODS

A BENCHMARKING SET OF MISSENSE VARIANTS

We collated a selective set of 170 missense variants from two online databases (Table 1), Human Cytochrome P450 (CYP) Allele Nomenclature Database (http://www.cypalleles.ki.se) and TPMT Alleles (https://www.imh.liu.se/tptmalleles?l=en), and cross-checked their functional effects against the predictions given by chosen algorithms.

These variants are strictly pharmacogenetic in that they have been shown to induce varied extents of changes in drug metabolism, but not manifest disease phenotypes (Apweiler et al. 2004). CYP enzymes serving a key physiological function, such as those that metabolise endogenous hormones, were excluded from our analysis. This was a criterion not included in previous work. For instance, Hao et al. (2010), in their analysis of phase II metabolising enzymes, did not exclude genes that had been implicated in the pathogenesis of a disease, such as UGT1A1. The gene encodes a protein that conjugates extraneous compounds and facilitates their removal; damaging UGT1A1 mutations are known to cause Gilbert Syndrome and familial neonatal hyperbilirubinaemia. Inclusion of pharmacogenetic variants with ambiguous properties could have affected the conclusions that they drew from their findings.

TABLE 1. Pharmacogenetic variants with known functional effects. These variants were compiled from two online databases, Human CYP Allele Nomenclature and TPMT Alleles.

| Gene    | Decreased- or null-function | Normal |
|---------|-----------------------------|--------|
| CYP1A1  | 1                           | 7      |
| CYP1A2  | 15                          | 12     |
| CYP2A6  | 5                           | 30     |
| CYP2B6  | 30                          | 5      |
| CYP2C8  | 1                           | 6      |
| CYP2C9  | 5                           | 13     |
| CYP2E1  | 1                           | 1      |
| CYP2F2  | 8                           | 6      |
| CYP3A4  | 2                           | 1      |
| CYP3A5  | 1                           | 1      |
| CYP4A11 | 2                           | 6      |
| CYP4F2  | 2                           | 2      |
| TPMT    | 33                          | 31     |
| Total   | 139                         | 31     |

PREDICTION OF FUNCTIONAL CONSEQUENCES

Eight prediction tools were tested (Table 2). They were selected based on their previously reported performance and distinct mechanisms that ranged from straightforward estimation of the evolutionary pressure imposed on a protein to merging various conservation and functional metrics into one prediction (Gray et al. 2012; Ingelman-Sundberg et al. 2018). Besides evaluating the performance of individual prediction tools, we also tested a consensus strategy whereby agreement between at least three tools was used as the basis for qualifying a DNA variant as
TABLE 2. Prediction tools that were benchmarked. These tools were selected based on their previously reported performance and also the relevance of their underpinning algorithms to the functional assessment of pharmacogenetic variants

| Prediction tool | Description | Input and output | Web server address |
|-----------------|-------------|------------------|--------------------|
| **Combined Annotation Dependent Depletion (CADD)** | CADD integrates 63 annotations - conservation and structural metrics - to produce a C-score that can be used to rate the deleteriousness of a DNA variant (Kircher et al. 2014) | **Input:** Nucleotide change and its genomic position  
**Output:** The C-score ranges from 1 to 99, where a higher score indicates a greater degree of pathogenicity | The C-scores were obtained from an online tool for variant annotation, SeattleSeq Annotation 138 (http://snp.gs.washington.edu/SeattleSeqAnnotation138/) |
| **Sorting Intolerant From Tolerant (SIFT)** | Based on multiple alignment of related proteins, SIFT determines the types of residues that are tolerated at a particular position. Infrequent amino acid changes are more likely to be deleterious (Ng & Henikoff 2003) | **Input:** Amino acid switch and an Ensembl identifier of the affected protein  
**Output:** The predictions follow a binary classification scheme and can be damaging or tolerated  
**Additional consideration:** For the prediction to be accurate, the selected protein sequences ought to be sufficiently diverse. In other words, alignment of very similar protein sequences would produce a false impression of a residue being highly conserved. The diversity of the aligned protein sequences is gauged by the median information content, which is provided for each prediction | http://sift.jcvi.org/ |
| **Polymorphism Phenotyping v2 (PolyPhen-2)** | PolyPhen-2 is similar to SIFT in that it also considers the conservation of a residue across related proteins to measure the impact of an amino acid substitution (Adzhubei et al. 2010). But, PolyPhen-2 also examines several other features such as protein structure and functional domains documented in the Pfam database | **Input:** A protein accession number, obtained from the Universal Protein Resource Knowledgebase (UniProtKB), and the amino acid substitution  
**Output:** PolyPhen-2 predictions are classified as follows: possibly damaging, probably damaging, or benign  
**Additional consideration:** PolyPhen-2 predictions are derived from two training data sets, HumDiv and HumVar. The data sets differ chiefly in how innocuous DNA variants are defined. In HumDiv, cross-species DNA differences are deemed harmless whereas in HumVar, high-frequency DNA variants (>1%) are considered non-damaging. Predictions based on either of the data sets are sometimes contrasting | http://genetics.bwh.harvard.edu/pph2/ |
| **Envision** | Envision gauges the underlying molecular effect of a DNA variant (Gray et al. 2018). Envision predictions are derived from deep mutational scanning, where cells expressing the wild-type or mutant protein are subjected to selection pressure, and the frequency of the variant in the resultant cell population is then determined by high-throughput DNA sequencing (Fowler & Fields 2014). Low-frequency DNA variants are probably damaging | **Input:** A UniProtKB protein identifier and the amino acid change  
**Output:** The output score ranges from 0 to 1. Wild-type variants are assigned a score of ~1. Envision scores >1 are possible and suggest increased enzyme activity. Lower scores indicate decreased protein function  
**Additional consideration:** Envision seems particularly suited for pharmacogenetic variants, whose phenotype may be obscure and hard to detect by purely in silico algorithms | https://envision.gs.washington.edu/shiny/envision_new/ |
| **Prediction tool** | **Description** | **Input and output** | **Web server address** |
|---------------------|----------------|---------------------|------------------------|
| **Site Directed Mutator (SDM)** | SDM computes free energy differences between wild-type and mutant proteins. The calculation is driven by the principle of *conformationally constrained substitution*. An amino acid residue can be safely substituted if the surrounding environment is a ‘fit’ for the alternate residue without having to undergo conformational changes (Pandurangan et al. 2017; Worth et al. 2011) | *Input*: Amino acid change and the three-dimensional structure of the affected protein, which can be obtained from the RCSB Protein Data Bank  
*Output*: Negative energy differences suggest reduced structural stability and that the mutant protein favours the unfolded state. Additional consideration: Where possible, we based the prediction on the native structure of a protein without a bound substrate. When there were multiple polypeptide chains in a protein, only the prediction inferred from the structure of chain A was included in our analysis | http://marid.bioc.cam.ac.uk/sdm2/ |
| **PANTHER-PSEP (Protein Analysis Through Evolutionary Relationships-Position-Specific Evolutionary Preservation)** | PANTHER bases its prediction on the evolutionary preservation of an amino acid residue. In lieu of measuring the cross-species or -homolog conservation of an amino acid residue, PANTHER reconstructs the ancestral sequence of a protein and computes how long a residue has remained unchanged in the protein over time (Tang & Thomas 2016). Highly preserved amino acids are less amenable to substitution | *Input*: Amino acid substitution and the FASTA sequence of the affected protein  
*Output*: PANTHER predictions are classified into *probably damaging*, *possibly damaging*, and *probably benign*. The preservation time of a DNA variant is also given | http://www.pantherdb.org/tools/csnpScoreForm.jsp |
| **Mutation Assessor** | Mutation Assessor performs an exhaustive assessment of the evolutionary pressure imposed on an amino acid. Protein homologs are first grouped into families and subfamilies to represent specific functional classes. Then, the importance of an amino acid is estimated by how often it is shared among members of a protein family or subfamily (Reva et al. 2011) | *Input*: Amino acid substitution and a UniProtKB protein identifier  
*Output*: The functional impact score (FIS) of a variant can be *neutral*, *low*, *medium*, or *high*. In this work, variants with a neutral or low FIS were deemed benign | http://mutationassessor.org/r3/ |
| **MutPred2** | MutPred2 differs from many tools in that it predicts the plethora of molecular changes caused by a DNA variant (Pejaver et al. 2017); the changes may affect the structure, domain, or function of a protein, such as its capacity to bind metals or macromolecules, allostery, catalytic activity, and posttranslational modification | *Input*: Amino acid change and the FASTA sequence of the affected protein  
*Output*: A score alongside predicted molecular impact. Significant changes are indicated by a p-value <0.05. Additional consideration: To reduce overlap between the tools, we only considered DNA variants with predicted molecular effect as *damaging* | http://mutpred.mutdb.org/index.html |
damaging (Frousios et al. 2013). The accuracy of a prediction tool was defined by the following formula:

\[
\frac{a + b}{2}
\]

where \(a\) and \(b\) were the fractions of the correctly identified damaging and neutral variants, respectively.

**STATISTICAL ANALYSIS**

Receiver operating characteristic (ROC) analysis was performed using easyROC (Goksuluk et al. 2016). Youden Index was chosen as the method for estimating optimal cut points.

**RESULTS AND DISCUSSION**

We first evaluated the diagnostic performance of CADD, Envision, and SDM, as the tools had not been tested on pharmacogenetic variants. ROC analysis showed that CADD and Envision, but not SDM, were reasonably accurate in detecting variants that affected pharmacogenes, yielding areas under the curve of 0.73 and 0.75, respectively (Figure 1 & Table 2). The optimal discriminating C-score was 8.34, appreciably lower than that recommended for disease-causing variants (Kircher et al. 2014). A large fraction of the damaging variants (89.21%) received a C-score >8.34 and were therefore correctly identified; however, many neutral variants were also misclassified (43.75%). Envision was much less sensitive than CADD, and a subthreshold score <0.86 pinpointed only 54.68% of the damaging variants. The drawback was offset by a relatively low rate of false positives (12.5%). Of the three methods, SDM was the worst-performing. It was unable to distinguish variants with differing functional impact (Table 2), suggesting that diminished stability was not a major attribute of mutant drug-metabolising proteins.

We then compared the performance of CADD, Envision, and SDM with other predictive algorithms, which are established tools in the functional assessment of diverse types of DNA variants (Chan 2013; Flanagan et al. 2010; Hao et al. 2011, 2010; Valdmanis et al. 2009; Zou et al. 2011). What set our work apart from previous analyses was that we used a narrow definition of pharmacogenetic variants and excluded those that affected CYP-mediated metabolism of crucial endogenous substrates. For instance, \(CYP21A2\) encodes an enzyme involved in the production of adrenal hormones. Inactivating \(CYP21A2\) variants cause congenital adrenal hyperplasia (Yu et al. 2011). Also, many non-\(CYP\) pharmacogenes have been implicated in the development of human diseases (Gottesman & Ambudkar 2001).

![FIGURE 1. Receiver operating characteristic analysis of CADD, Envision, and SDM scores for defining functionally relevant pharmacogenetic variants](image_url)

**TABLE 3. Receiving operating characteristic analysis**

| Method | Area under the curve (± SE*) | p value | Optimal cut-off | Youden’s optimal criterion |
|--------|-----------------------------|---------|----------------|---------------------------|
| CADD   | 0.73 ± 0.06                 | 5 × 10⁻³| 8.34           | 0.45                      |
| Envision | 0.75 ± 0.05               | 0       | 0.86           | 0.42                      |
| SDM    | 0.58 ± 0.06                 | 0.17    | -0.58          | 0.24                      |

*Standard error
FIGURE 2. The percentages of decreased- or null-function variants and neutral variants correctly identified by the prediction methods benchmarked in this work. A consensus strategy, whereby agreement between at least three predictive algorithms was required for qualifying a DNA variant as damaging, was found to outperform other methods. Note that PolyPhen-2 emits sometimes contrasting predictions based on either of its training data sets, HumDiv and HumVar.
Although CADD and Envision are touted as having a thorough prediction scheme, they have not outperformed SIFT, Mutation Assessor, or PolyPhen-2 in our analysis (Figure 2). Of the eight tested tools, SIFT gave the best performance despite its simple algorithm; its accuracy reached 75.65%. Mutation Assessor followed SIFT closely with an accuracy rate of 74.53%. It is worth noting that SIFT and Mutation Assessor have very similar algorithms (Table 2). Both methods derive their predictions from gauging the persistence of important amino acids throughout evolution; therefore, they may tend to underestimate the functional impact of pharmacogenetic variants. Indeed, we found that SIFT was better at predicting the neutrality of pharmacogenetic variants than their deleteriousness (Figure 2). Our result concurs with that of a previous study showing that pharmacogenetic variants were almost indistinguishable from harmless DNA changes that occurred throughout the human genome (Li et al. 2014).

In previous analyses, PolyPhen-2, or its older version, was often noted to surpass SIFT in detecting damaging DNA variants (Chan 2013; Flanagan et al. 2010; Gray et al. 2012). However, we found that PolyPhen-2 performed less well than expected. PolyPhen-HumVar correctly identified 66.91% of the decreased- or null-function variants and 78.13% of the innocuous variants. PolyPhen-HumDiv was better at predicting damaging variants but fared worse against neutral variants (72.66% vs. 68.75%). Both models detected fewer neutral variants than did SIFT, lowering their overall accuracy. The discrepancy between our result and those of previous work could be ascribed to the different variant sets that were used to benchmark the tools.

Overall, none of the tested tools struck a balance between the rates of true positives and false positives (Figure 2). Therefore, we trialled a consensus approach whereby the prediction of functional relevance was based on agreement between at least three methods. This approach attained a high degree of sensitivity (84.17%) without yielding an unacceptable rate of false positives (29.03%). The impact of DNA variants could arise from disparate mechanisms, the assessment of which requires a multipronged approach. The complex effect of DNA variants may explain why the consensus strategy performed better than other methods used alone. A deleterious variant might evade detection by some tools but not others. Future work may benefit from a detailed analysis of pharmacogenetic variants to pinpoint the features that contribute most substantially to their damaging consequences. For consensus prediction, the included tools may be carefully chosen to optimise its accuracy. Moreover, each tool may be assigned a weighting according to its mechanism of prediction. For instance, the superior performance of SIFT and Mutation Assessor suggests that pharmacogenetic variants may experience weak yet significant evolutionary pressure; hence, these methods can be given greater importance when combined with others to predict the functional effect of a pharmacogenetic variant (Ingelman-Sundberg et al. 2018).

STUDY LIMITATIONS
Our study has several limitations that should be considered. First, in our benchmarking set of variants, the damaging variants far outnumbered the neutral variants. A slight change in the number of correctly predicted neutral variants would alter the rate of true negatives appreciably. Second, we assumed that the influence of a DNA variant on enzyme function is consistent across different substrates. This contradicts prior observations that some pharmacogenetic variants enhanced the action of an enzyme on some of its substrates while decreasing its activity towards other substrates (Sakuyama et al. 2008; Yu et al. 2002). Third, we did not iteratively test the effect of an amino acid substitution in each polypeptide that comprises a protein. Instead, we assumed that the amino acid change in any of the polypeptides should produce a similar effect on the structural stability of the protein.

CONCLUSION
Accurately predicting the functional impact of a DNA variant is a long-standing conundrum in genomics research. Disagreement between algorithms is not uncommon, and the performance of a tool is known to vary across variant types (Flanagan et al. 2010; Mather et al. 2016). These problems could be more severe in the analysis of pharmacogenetic variants, as the current predictive algorithms are not trained for gauging non-pathogenic deleteriousness. Thus, assessing the efficacy of different algorithms in deciphering the functional impact of pharmacogenetic variants formed the basis of our work.

We have determined that the top-performing prediction tools, when benchmarked against a set of strictly defined pharmacogenetic variants, were SIFT, Mutation Assessor, and CADD. However, none of them struck an optimal balance between sensitivity and specificity. The accuracy of prediction was highest (77.57%) when we adopted a consensus strategy whereby the deleteriousness of a variant was defined by agreement between at least three methods. Therefore, future investigations may focus on designing a comprehensive method for assessing the functional relevance of pharmacogenetic variants, by combining a variety of carefully chosen tools and assigning each of them a weighting based on their mechanism of prediction. The development of the method should be facilitated by the expanding data sets of well-annotated pharmacogenetic variants.

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