Automated inference of molecular mechanisms of disease from amino acid substitutions

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ABSTRACT
Motivation: Advances in high-throughput genotyping and next generation sequencing have generated a vast amount of human genetic variation data. Single nucleotide substitutions within protein coding regions are of particular importance owing to their potential to give rise to amino acid substitutions that affect protein structure and function which may ultimately lead to a disease state. Over the last decade, a number of computational methods have been developed to predict whether such amino acid substitutions result in an altered phenotype. Although these methods are useful in practice, and accurate for their intended purpose, they are not well suited for providing probabilistic estimates of the underlying disease mechanism.

Results: We have developed a new computational model, MutPred, that is based upon protein sequence, and which models changes of structural features and functional sites between wild-type and mutant sequences. These changes, expressed as probabilities of gain or loss of structure and function, can provide insight into the specific molecular mechanism responsible for the disease state. MutPred also builds on the established SIFT method but offers improved classification accuracy with respect to human disease mutations. Given conservative thresholds on the predicted disruption of molecular function, we propose that MutPred can generate accurate and reliable hypotheses on the molecular basis of disease.

Availability: http://mutdb.org/mutpred
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1 INTRODUCTION
With rapid advances in high-throughput genotyping and next-generation sequencing technologies, a vast amount of genetic variation has been discovered and deposited in databases, with much more still to come (Mooney, 2005). Currently, there are over 50,000 coding region mutations, causing or associated with human genetic disease, listed in the Human Gene Mutation Database (HGMD) (http://www.hgmd.org; Stenson et al., 2009), of which ~40,000 are amino acid substitutions. Amino acid substitutions have the potential to alter the function of their corresponding protein, either directly or via disruption of structure. Hence they are of particular interest as candidates for further experimental assessment (Cargill et al., 1999; Ng and Henikoff, 2006).

There is a need to effectively and efficiently identify functionally important variants which may be deleterious or disease-causing and to identify their molecular effects. For this purpose, a number of computational methods, based on amino acid sequence, structure and evolutionary information, have been proposed (Hon et al., 2008; Karchin, 2009; Mooney, 2005; Steward et al., 2003). One of the earliest tools developed in this area, Sorting Intolerant From Tolerant (SIFT), uses sequence homology to classify amino acid substitutions as tolerated or deleterious (Ng and Henikoff, 2001, 2003). Owing to its impressive predictive power and simplicity, SIFT continues to be used as a benchmark for other methods and approaches (Bao and Cui, 2005; Bromberg and Rost, 2007; Chan et al., 2007; Kulkarni et al., 2008). Protein structure information has been incorporated through the rule-based approach of Wang and Moult (2001) and supervised methods by Yue et al. (2005) and Yue and Moult (2006). PolyPhen also exploits sequence conservation, structure, and rich annotations from protein databases to classify damaging amino acid substitutions (Ramensky et al., 2002; Sunyaev et al., 2001). Scores utilizing more sophisticated alignments based on hidden Markov models from protein families were incorporated with the development of the Substitution Position-Specific Evolutionary Conservation (subPSEC) scores in the PANTHER database (Thomas et al., 2003). Features from comparative protein structural models have been incorporated via the LS-SNP approach (Karchin et al., 2005), while the usefulness of ab initio structural models was evaluated by Saunders and Baker (2002). Finally, servers such as SNAP (Bromberg and Rost, 2007), PMUT (Ferre-Roja et al., 2005), or CanPredict (Kaminker et al., 2007a, b) use a number of sequence-based, structural and evolutionary features, combined with various classification approaches and training sets.
Currently, there are three general areas in need of improvement for the development of better classification approaches. First, experimentally validated and unbiased training sets of disease causing or deleterious mutations need to be developed. Ideally, these data sets would be quantitatively phenotyped at both the organismal and molecular level. Second, new mutational attributes (i.e. beyond sequence composition, structure and evolutionary conservation) that improve classification accuracy and yield hypotheses on molecular mechanisms of disease, need to be identified and characterized. Finally, new computational approaches need to be developed that improve classification accuracy when similar attributes and training sets are used. The second and third areas are the focus of this work.

Although current approaches are valuable in selecting and prioritizing mutations by yielding the most likely disease-causing proteins (one of the major goals of the HSTTT, and a few of them provide clues as to the putative molecular mechanisms by which the mutations affect phenotypes (Wang and Moult, 2001; Yue et al., 2005; Yue and Moult, 2006), and even fewer when protein structure is not available. This is a severe limitation since it is unreasonable to expect that a high-resolution 3D structure will be available for all proteins and their mutants, either due to difficulties in structure determination and modeling, or because the proteins are intrinsically disordered (Dunker et al., 2001). Therefore, introducing novel sequence-based approaches is important.

Several systematic studies have shown that predicting the molecular underpinnings of disease is feasible in practical terms: for example, by comparing the crystal structures or 3D models between wild-type and mutant proteins, Wang and Moult (2001) were able to catalog amino acid substitutions into five classes according to their effects on molecular function. These classes included changes in protein stability, but also direct changes in protein function via disrupted ligand binding, catalysis, allosteric regulation, and post-translational modifications, without necessarily affecting the stability of the molecule. Gains of N-linked glycosylation sites causatively implicated in disease were studied by Vogt et al. (Vogt et al., 2005, 2007). In our previous work, mutations predicted to cause a gain or loss of phosphorylation target sites were found to be significantly more prevalent among somatic cancer mutations than in control data sets, suggesting that phosphorylation target site mutation is an active general mechanism of dysregulation in cancer (Radivojac et al., 2008).

In this study, we extend our approach to include a number of additional structural and functional properties and show that predictions of the gain and loss of such properties can provide good classification accuracy, but also have the potential to estimate, in probabilistic terms, the underlying biochemical cause of disease.

2 METHODS

2.1 Data sets

A collection of five data sets of human amino acid substitutions were constructed from online databases and the literature (Table 1). Four of these data sets are composed of disease-associated mutations (CANCER, Kinase, HMD and SPp), whereas the remaining data set (SPd) contains inherited, putatively neutral polymorphisms. The CANCER data set comprises somatic mutations in genes re-sequence from 22 cell lines from breast and colorectal cancer tissues (Sjoblom et al., 2006). The Kinase data set contains somatic mutations from sequencing kinase genes from 210 individual tumors (Greenman et al., 2007). Both of these sets are expected to contain mutations that lead to neoplastic progression (drivers) as well as neutral mutations that do not influence tumorigenesis (passengers). Mutations annotated with evidence for being disease-causing in HGMD constitute inherited disease-causing mutations in the HSTTT data set. Finally, the Swiss-Prot database (Boccahmann et al., 2003) contains amino acid substitutions that are either disease-causing (SPp) or polymorphic (SPd). The full sequences of proteins harboring these mutations were downloaded and used for attribute generation. Table 1 describes the data sets, excluding the substitutions for which at least one of the attributes could not be computed.

<table>
<thead>
<tr>
<th>Data set</th>
<th>Substitutions</th>
<th>Proteins</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>CANCER</td>
<td>653</td>
<td>519</td>
<td>Disease, somatic</td>
</tr>
<tr>
<td>Kinase</td>
<td>422</td>
<td>231</td>
<td>Disease, somatic</td>
</tr>
<tr>
<td>HMD</td>
<td>26 655</td>
<td>1305</td>
<td>Disease, inherited</td>
</tr>
<tr>
<td>SPd</td>
<td>6606</td>
<td>680</td>
<td>Disease, inherited</td>
</tr>
<tr>
<td>SPp</td>
<td>23 426</td>
<td>8728</td>
<td>Neutral, inherited</td>
</tr>
</tbody>
</table>

Table 1. Summary of data sets

2.2 Attribute construction

We generated a broad array of attributes from protein sequences and utilized them in classification. These attributes can be grouped into three classes: (i) attributes based on predicted protein structure and dynamics, (ii) attributes based on predicted functional properties and (iii) attributes based on amino acid sequence and evolutionary information. Sequence-based predictions of structural features or protein dynamics included secondary structure (Rost, 1999), solvent accessibility (Rost, 1996), transmembrane helices (Kosuge et al., 2001), coiled-coil structure (DeLerrnan and Speed, 2002), stability (Capritti et al., 2005), B-factor (Radivojac et al., 2004), and intramolecular disorder (Peng et al., 2006). Predictions of functional sites included DNA-binding residues (Ahmad et al., 2004), catalytic residues (Xin and Radivojac, unpublished data), calmodulin-binding targets (Radivojac et al., 2006), as well as the phosphorylation (Iakoucheva et al., 2004), methylation (Daily et al., 2005), ubiquitination (Radivojac et al., 2009) and glycosylation (Radivojac, unpublished data) sites. Note that the calmodulin-binding target classifier predicts short structured or loosely structured helical segments within otherwise disordered regions and was used as a substitute for predicting Molecular Recognition Fragments (MoRFs), as proposed by Mohan et al. (2006). Each predictor, with the exception of N-linked glycosylation, was constructed in a supervised learning scenario, whereas the changes of N-linked glycosylation were modeled by simply counting the changes of N-linked glycosylation, was constructed in a supervised learning scenario, whereas the changes of N-linked glycosylation were modeled by simply counting
Table 2. Structural and functional properties used by MutPred

<table>
<thead>
<tr>
<th>Structural properties</th>
<th>Functional properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secondary structure</td>
<td>DNA-binding residues</td>
</tr>
<tr>
<td>Solvent accessibility</td>
<td>Catalytic residues</td>
</tr>
<tr>
<td>Stability</td>
<td>MemRPs</td>
</tr>
<tr>
<td>Intrinsic disorder</td>
<td>Phosphorylation sites</td>
</tr>
<tr>
<td>B-factor</td>
<td>Methylation sites</td>
</tr>
<tr>
<td>Transmembrane helix</td>
<td>Glycosylation sites</td>
</tr>
<tr>
<td>Coiled-coil structure</td>
<td>Ubiquitination sites</td>
</tr>
</tbody>
</table>

physically separate molecules, are considered independent. Similarly, the gain of structural or functional property \( p \) can be expressed as:

\[
P(\text{gain of property } p \text{ at } s_i) = (1 - P(p|s_i^\text{g})) \cdot P(p|s_i^\text{g}).
\]

It is clear from Equations (1) and (2) that the greater the difference between \( P(p|s_i^\text{g}) \) and \( P(p|s_i^\text{g}) \), the greater the probability of gain or loss of the property. However, note that a reduction of score from 1.0 to 0.9 corresponds to the probability of loss of 0.1, whilst the reduction of score from 0.5 to 0.4 corresponds to the probability of the loss of property of 0.3.

The gain and loss of a property at residue \( s_i \) does not necessarily suggest that the amino acid substitution occurred at position \( i \). This situation is particularly interesting for single-residue functional sites such as post-translational modifications, because impacts of substitutions of neighboring residues cannot be easily detected even if the functional site is known. We refer to such cases as functional neighborhood mutations, whereas the direct changes of functional sites are referred to as functional site mutations. In general, we expect that the probability of gain or loss of function at \( s_i \) will be inversely correlated with the distance of the substitution site from \( s_i \) (here we consider residues between positions \( -5 \) and \( +5 \) from the substitution site).

Thus, the largest impact on protein function is likely to be for the functional site mutations. In the case of a loss of property, this results in \( P(p|s_i^\text{g}) = 0 \) and therefore the probability that the wild-type sequence is functional at \( s_i \) equals the probability that the function will be lost. An example of such a situation is when a phosphorylatable serine residue is substituted by a non-phosphorylatable residue such as alanine. Similarly, in the case of a gain of function, the probability that \( s_i \) is non-functional in the wild-type equals 1, i.e. \( P(p|s_i^\text{g}) = 0 \). Hence, for functional site mutations, the prediction of protein property in the mutated and wild-type protein also predicts the gain or loss of that property, respectively. In total, seven structural and seven functional properties were used in this study (Table 2).

2.4 Performance evaluation

To measure the ability of classifiers to discriminate between disease and neutral substitutions, we plotted receiver operating characteristic (ROC) curves and calculated the area under the ROC curve (AUC). ROC curve shows the true positive rate (or sensitivity, \( TPR \)) as a function of the false positive rate. The false positive rate is typically denoted as \( 1 - FPR \), where specificity (\( 1 - FPR \)) is the accuracy on the negative data points. In this study, disease-associated mutations were considered to be positive examples, whereas the neutral polymorphisms were negative. For the classification using Cancer data set, the set of neutral polymorphisms was constructed by retaining only those substitutions found in cancer-associated proteins, as provided by the Cancer Gene Census (Futreal et al., 2004). Similarly, only kinases from the BRENDA database (Chang et al., 2009) were used to select a subset of neutral polymorphisms in evaluating the performance on KINASE. In total, the number of neutral polymorphisms from the Cancer and KINASE data sets was 1625 (480 proteins) and 1803 (394 proteins), respectively. Classification models were evaluated using per-protein 10-fold cross-validation.

2.5 Predicting molecular mechanism of disease

Owing to unknown class priors, it is not possible to estimate the precision of most structural and functional predictors accurately. Thus, our predictions of molecular mechanisms of disease are based upon the assumption that the majority of phenotypically neutral polymorphisms are unlikely to affect protein structure or function significantly. In such a situation, a distribution of scores can be created for each gain and loss of property using the data set SPV (or its filtered versions in the cases of Cancer and KINASE data). Then, each gain or loss score \( sc \) from Equations (1) and (2) of a disease mutation can be assigned a \( P \)-value, i.e. the probability that a randomly selected neutral polymorphism will have the same score \( sc \) or higher. We refer to such \( P \)-values as property scores.

3 RESULTS

3.1 Discrimination between disease and neutral mutations using machine learning

The performance of individual classifiers is summarized in Table 3. For each classifier, we report sensitivity, specificity, accuracy and the area under the ROC curve. To alleviate the potential negative influence of unbalanced data between disease and neutral polymorphisms on classification performance, we also trained classifiers on equal-sized disease and neutral sets. We found that balancing the training data had almost no effect on the area under the ROC curve, but that it slightly impacted the classification accuracy. Since SIFT is an established method for distinguishing deleterious from putatively neutral polymorphisms and is easily portable, we used it for benchmarking. Thus, the AUC of SIFT scores provide our baseline measure.

Unsurprisingly, SIFT scores alone worked well on the HOMD, and SPV data sets (Table 3). The relatively inferior performance of SIFT on Cancer and KINASE data suggests large differences in evolutionary conservation between the amino acid residues harboring inherited and somatic mutations. The performance of other classifiers also resulted in a relatively low accuracy on the Cancer and KINASE data sets; this may be due to the fact that somatic mutations in cancer are likely to contain a large number of so-called passenger mutations (Futreal et al., 2005).

The AUCs of the SVM across all data sets, were 3.0 percentage points greater than those of SIFT, suggesting that a linear SVM had a limited ability to extract useful information from additional features.
Table 3. Performance accuracy of different classification models on four data sets containing disease-associated amino acid substitutions versus the data set of inherited polymorphisms

<table>
<thead>
<tr>
<th>Data set</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Accuracy (%)</th>
<th>AUC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SIFT</td>
<td>SVM</td>
<td>RF</td>
<td>SIFT</td>
</tr>
<tr>
<td>CANCER</td>
<td>50.8</td>
<td>43.1</td>
<td>57.5</td>
<td>67.1</td>
</tr>
<tr>
<td>KSASE</td>
<td>51.7</td>
<td>37.6</td>
<td>55.1</td>
<td>61.8</td>
</tr>
<tr>
<td>HGMD</td>
<td>70.8</td>
<td>75.6</td>
<td>72.6</td>
<td>73.9</td>
</tr>
<tr>
<td>SPo</td>
<td>74.1</td>
<td>77.6</td>
<td>76.5</td>
<td>73.9</td>
</tr>
</tbody>
</table>

Compared are SIFT, a linear SVM and a RF approach using 1000 trees. Bold fonts indicate the best performing models ($P < 0.001$ in each pairwise comparison with SIFT; t-test based on 10 repeats).

3.2 Assessing the molecular mechanism of disease

Since there are relatively few amino acid substitutions for which the molecular mechanism of disease is known, it is not currently possible to directly evaluate the accuracy of approaches designed to predict such mechanisms. Thus, our approach relies on the postulate that inherited polymorphic sites have little or no influence on the structure and function of proteins. Under this assumption, one can generate a distribution of scores for each gain or loss of property over neutral polymorphisms and output the $P$-value as a quantitative score for each property prediction.
To investigate the influence of individual attributes on the sets of disease-causing mutations in Swiss-Prot, we generated for 11.6% of disease-causing mutations in Swiss-Prot, as well as 10.2 and 12.1% of somatic mutations in Kinase and Cancer data sets. Finally, we note that very confident hypotheses can be generated for 5.5% of mutations in both HGMD and Swiss-Prot, and for 1.2% of substitutions in somatic mutation data sets.

3.3 Importance of gain/loss of properties on inherited and somatic amino acid substitutions

To investigate the influence of individual attributes on the sets of inherited and somatic mutations, we compared the most common actionable hypotheses of the molecular mechanism of disease in the HGMD, Cancer, and Kinase data sets. We selected HGMD as a representative of the set of inherited mutations owing to its large size and the fact that most of the mutations from SPs are already listed in HGMD (79%).

The most common actionable hypotheses that characterized inherited, but not somatic mutations, were order-to-disorder transition (10.2% of all actionable hypotheses in HGMD, 2.7% in Cancer, 3.7% in Kinase) and loss of stability (7.1% in HGMD, 2.5% in Cancer, 1.3% in Kinase), thereby emphasizing the disruption of structure in monogenic disease. By contrast, Cancer and Kinase data were enriched in the disruption of post-translational modifications (25.0% in HGMD, 31.1% in Cancer, 39.0% in Kinase). Using a complete set of 30 categories, we determined that the differences between the HGMD and Cancer (\(P = 5.0 \times 10^{-5}; \chi^2\) test) and Kinase (\(P = 5.0 \times 10^{-5}; \chi^2\) test) data sets were statistically significant. Figure 3 shows the most significant differences among the three sets.

To additionally validate the strength of gain and loss of structural/functional properties, we trained a prediction model based only on these attributes with the goal of discriminating between amino acid substitutions on which the overall model had score \(>0.5\) and neutral substitutions. The area under the ROC curve using these attributes ranged between 71.0% (SPO) and 79.1% (HGMD), indicating, somewhat surprisingly, that the gain and loss of structural/functional properties alone have good discriminatory power and are suitable for the purpose of assessing the mechanism of disease.

In Figure 4, we show the relative ranking of various attributes on different training data, using the gini index. Loss and gain of structural and functional properties are indicated by \(\times\)'s, whereas SIFT is represented by a black triangle. There were significant differences between the inherited and somatic amino acid substitutions. In the somatic mutations data sets, the gain/loss of most structural/functional properties were higher ranked in Cancer and Kinase than in HGMD (presented as \(\times\)'s below the diagonal line).

3.4 Case studies

We searched the literature and found direct experimental evidence for several disease-causing mutations with a high score for a gain or loss of structural or functional properties. The first example is phosphatase and tensin homolog (gene name: PTEN), a tumor suppressor gene that negatively regulates the AKT/PI3K signaling pathway. PTEN acts as both dual-specificity protein phosphatase and lipid phosphatase that is considered to be critical for its suppressor function. Many PTEN gene mutations were identified and associated with classical Cowden syndrome (CS), Bannayan-Riley-Ruvalcaba syndrome, Proteus syndrome and Proteus-like syndrome (Eng, 2003). PTEN mutations, especially those located in exons 5, 7 and 8, have been found in 80% of CS patients (Marsh et al., 1998, 1999). Exon 5, which encodes the phosphatase core motif, accounts for 40% of all PTEN mutations (Eng, 2003) and includes catalytic residues C124 and D92 (Lee et al., 1999). Substitutions C124R and D92E, listed in HGMD, are associated with Cowden syndrome and are known to affect PTEN’s phosphatase function (Eng, 2003). The MutPred general score for C124R was 0.64 (SIFT score was 0; SIFT predictions below 0.05 are considered positive) and the catalytic residue predictor yielded a loss of property prediction of 0.57, which resulted in the property score \(P = 0.048\) for the loss of catalytic residue propensity. Substitution D92E, on the other hand, was attributed a MutPred score of 0.98 (SIFT score 0) and a loss of catalytic residue score of 0.42 (\(P = 0.18\)). Although D92E is not considered an actionable hypothesis by our criteria, its strong score may serve as an indicator that the thresholds selected in Section 3.3 are stringent.

The second example involves human PTP synthase (gene name: PTS), a carbon-oxygen lyase that catalyzes triphosphate elimination yielding 6-pyruvoyltetrahydrobiopterin. PTP synthase has 19 documented amino acid substitutions in Swiss-Prot release 56.6 and defects in PTP synthase are known to be associated with hyperphenylalaninemia (HPA). HPA is an autosomal recessive disorder with serious neurological symptoms that represents a mild
form of phenylketonuria. The mutation R16C is documented to diminish phosphorylation of S19 by PKG and also to cause HPA (Oppliger et al., 1995; Scherer-Oppliger et al., 1999; Thomy et al.,
1994). The MutPred general score for this mutation is 0.87 (SIFT score 0.01) whereas the phosphorylation score for S19 decreased from 0.85 to 0.49 (loss of phosphorylation score of 0.43; P = 0.006 over all functional neighborhood mutations; P = 0.058 over all functional site and neighborhood mutations) upon introducing mutation R16C, as predicted by DisPhos (Iakoucheva et al., 2004). Probabilistically quantifying such a reduction in phosphorylation likelihood is difficult even if it is known that S19 is a phosphorylation site. Therefore, the use of computational models is highly important.

4 DISCUSSION

Here we introduced and evaluated a new computational model that builds upon SIFT by explicitly estimating probabilities of affecting various structural and functional properties, such as the loss of helical propensity, catalytic activity or post-translational modifications. Over the last decade, several methods have been proposed and tested to predict whether a particular amino acid substitution affects protein function leading to an altered phenotype. These approaches are reasonably accurate and useful. However, they do not generate hypotheses relating to the biochemical cause of disease. In our model, the loss and gain of each structural and functional property was directly modeled via posterior probabilities, thereby enabling us to directly estimate the contribution of a gain/loss of a given property in order to deduce the underlying mechanism of disease. In this way, our method indirectly exploits the structural and functional data available for functional prediction, effectively enlarging the training data sets beyond the characterized disease-causing events.

Attributes representing predictions of gain/loss of structural and functional properties also contributed to an improved classification performance over SIFT. The increase in classification performance ranged between 5.9 (Hgmd) and 7.8 (Kinase) percentage points. Hsmd was eventually used to train the final model, MutPred. The performance of random forest algorithms was better than that of support vector machines, probably due to the explicit modeling of the gain/loss of structural and functional properties which can be more easily exploited by decision trees.

The good prediction accuracy of MutPred on Cancer and Kinase data indicates that somatic sites can be predicted when compared to inherited polymorphisms, even when the final model was trained on Hsmd data alone (data not shown). This was not surprising since the amino acid residues which harbor somatic mutations are expected to be under a different set of evolutionary constraints than those harboring inherited polymorphisms. However, we believe that using molecular features to identify causative somatic mutations (drivers) may be more difficult than for inherited mutations because passenger mutations can introduce or disrupt functional sites even although they may not exert an effect within the context of a particular cell or tissue type. For example, amino acid substitutions in a kinase catalytic domain that is not expressed could be predicted to be damaging, but would in practice have no observable phenotypic effect since the protein product would not be present in the cell. To allow for tissue differences and particular predicted molecular events, future improvements in the classification models should incorporate more detailed information on the particular context of disease.

Based on sample data from the Protein Data Bank, Wang and Moult (2001) developed a rule-based approach to characterize molecular causes of disease. They also provided first assessments of the ways in which disease-causing mutations might affect protein function: >80% of inherited mutations were estimated to disrupt protein stability as a proxy to changed function, whereas <10% could be attributed to direct changes of functional residues. Interestingly, using a very different approach, we arrived at similar conclusions, with the advantage of being able to predict such events from sequence. Our results are also in agreement with the study of Torkamani and Schork (2007), who achieved improved prediction accuracy on a kinase-specific data set compared to SIFT. Furthermore, we find that somatic mutations, although predictable, may affect cellular functions in ways that are subtler and more diverse than for inherited disease mutations.

In conclusion, we used the most comprehensive data set of disease-associated mutations and incorporated new attributes for classification that directly model the gain/loss of structural and functional properties. We believe that this type of probabilistic evidence is informative and complements evidence that a conserved residue is disrupted.

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Conflict of Interest: none declared.

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