Protein Science
DOI 10.1002/pro.555

Response to "PREDICTABLE DIFFICULTY OR DIFFICULTY TO PREDICT" by Tamás Arányi, Krisztina Fulop, Orsolya Symmons, Viola Pomozi, and András Váradi

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Using our automated bioinformatics tool, we recently classified 40 point mutations in ABC transporters as "neutral" or "disease-associated", followed by experimentally testing three of the predictions.[4] Scanning the scientific literature, Varadi *et al.* found support for 9 of our predictions, while suggesting that 10 were incorrect. However, the interpretation of the literature by Varadi *et al* is itself not unambiguous, as illustrated by at least 6 of the 10 examples of our presumed error.

The first five examples are likely pleiotropic mutations, with a different impact on a different function:

Mutation Q141K in the breast cancer resistance protein BCRP does not significantly influence the disposition of HIV drug lamivudine,[5] thus not supporting the unequivocal "disease-associated" classification by Varadi *et al*.

Mutation P269S in BCRP is associated with a 40% decrease in vesicular uptake of [(3)H]estrone-3-sulfate and [(3)H]methotrexate compared to the wild-type form,[8] which is inconsistent with the unambiguous "neutral" annotation.

Mutation V444A in the bile salt export pump BSEP does not change taurocholate transport function *in vitro*;[6] homozygous V444A mutations were prevalent in both progressive familial intrahepatic cholestasis patients and in non-cholestatic patients;[1] and this variant has also been observed in other healthy populations.[7] Thus, a clearcut classification as "disease-associated" is not justified.

Mutant V1251I of ABCB1 showed decreased transport of BODIPY-FL-paclitaxel, but increased transport of calcein-AM in an assay using transfected HEK293T cells,[2] arguing against an unequivocal "disease-associated" classification.

Mutation S1141T in ABCB1 showed either normal or increased transport in the yeast assay,[3] and increased function in transfected HEK293T cells.[2] Therefore, this mutation should not be classified simply as "disease-associated".

The final, sixth case is an example of the lack of evidence for the classification by Varadi *et al.*: Mutation A1291T in ABCC6 occurs simultaneously with a nonsense mutation (Y1069X) in the analyzed form of the transporter.[9] Therefore, we do not yet know whether the nonsense mutation or the A1291T mutation (or both) is causative of the disease state, contrary to a definitive "disease-associated" classification.

If one accepts the qualifications above, 5 of the 40 predictions by our automated tool are invalidated, including 1 by our experiments[4] and 4 by the scientific literature cited by Varadi *et al.*; and 11 mutations are confirmed, including 2 by our experiments[4] and 9 by the literature cited by Varadi *et al*. The corresponding accuracy level (69%) is in fact comparable to that estimated by the random forest test (Figure 4 in ref.[4]).

In conclusion, we agree with Varadi *et al* that there are inherent difficulties in predicting the functional impact of point mutations. Moreover, as we illustrate above, there are also inherent difficulties in characterizing the functional impact of mutations by experiment, and in interpreting the corresponding scientific literature. Classification of mutations into only two classes (such as

Received: Nov 03, 2010; Accepted: Nov 04, 2010

our “neutral” and “disease-associated” classes) is a serious limitation, given the complexities of protein localization, degradation, interactions with other small molecules and macromolecules, as well as function in general, resulting in the pleiotropy of some mutations.[2] Nevertheless, the difficulty of the problem does not mean we should not begin to address it as long as the progress is properly qualified, as we believe we did in our publication.

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