

TRANSLATIONAL RESEARCH

Comparison of pathogenicity prediction tools on missense variants in RYR1 and CACNA1S associated with malignant hyperthermia

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Abstract

Background: Malignant hyperthermia (MH) is a pharmacogenetic disorder that has been linked to the skeletal muscle calcium release channel (RYR1) and the α 1S subunit of the voltage-dependent L-type calcium channel (CACNA1S). Genomic DNA capture and next generation sequencing are becoming the preferred method to identify mutations in these genes. Bioinformatic pathogenicity prediction of identified variants may help to determine if these variants are in fact disease causing.

Methods: Eight pathogenicity prediction programmes freely available on the web were used to determine their ability to correctly predict the impact of a missense variant on RyR1 or dihydropyridine receptor (DHPR) protein function. We tested MH-causative variants, variants that had been shown to alter calcium release in cells, and common sequence variants in RYR1 and CACNA1S.

Results: None of the prediction programmes was able to identify all of the variants tested correctly as either 'damaging' (MH-causative variants, variants that had been shown to alter calcium release in cells) or as 'benign' (common sequence variants). The overall sensitivity of predictions ranged from 84% to 100% depending on the programme used, with specificity from 25% to 83%.

Conclusions: In this study we determined the sensitivity and specificity of bioinformatic pathogenicity prediction tools for RYR1 and CACNA1S. We suggest that the prediction results should be treated with caution, as none of the programmes tested predicted all the variants correctly and should only be used in combination with other available data (functional assays, segregation analysis).

Key words: bioinformatics; malignant hyperthermia; prediction; variants

Malignant hyperthermia (MH) is an autosomal dominant disorder (OMIM #145600) that affects skeletal muscle calcium release from the sarcoplasmic reticulum (SR) and has been linked to mutations in genes encoding the ryanodine receptor 1 (RYR1, 19q13.2) and the α 1S subunit of the skeletal muscle dihydropyridine receptor (CACNA1S, 1q32.1). The disease is triggered in susceptible individuals by exposure to inhalational anaesthetics and/or succinylcholine and results in tachycardia, hyperthermia and metabolic acidosis. The ryanodine receptor 1 (RyR1) is mainly expressed in the sarcoplasmic reticulum (SR) of skeletal muscle and

is a calcium release channel which is coupled to the dihydropyridine receptor in the T-tubule of the sarcolemma.

To date there have been more than 300 variants of unknown significance (VUS) associated with MH in RYR1, while only 35 have been shown to be causative of the disease. The European Malignant Hyperthermia Group (EMHG) guidelines state that in order to class a variant as causative, the variant needs to be genetically and functionally characterized.¹ Functional assays have been performed so far either *ex vivo* in patient cells (muscle and B lymphoblastoid cells from patients), or in recombinant systems

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Editor's key points

- Malignant hyperthermia (MH) has been linked to a number of gene variants.
- Other variants have been identified which may also be important for MH susceptibility.
- Functional characterization of variants is time consuming.
- Several online bioinformatics tools are available.
- None of the programmes correctly predicted known causative variants and should only be used alongside functional testing.

(muscle cells from dyspedic mouse, knock in mouse models or HEK293 cells). As the cost for targeted DNA sequencing is decreasing, targeted DNA capture combined with next generation sequencing (NGS) is becoming the preferred option for variant detection and is being used diagnostically. There are a vast number of identified VUS which may or may not be associated with a certain disease, and which need to be filtered.

This is a significant bottleneck in DNA-based diagnosis for MH because of the large size of the RYR1 gene, the large number of known variants and the difficulty involved with functional analysis. The ability to accurately predict the pathogenicity of a specific missense variant would aid diagnosis considerably, and prevent MH episodes, along with avoiding the muscle biopsy and *in vitro* contracture test (IVCT). There are many bioinformatic tools freely available that allow pathogenicity prediction of VUS and researchers are using them to predict the effect of amino acid changes in RyR1 on protein function.²⁻⁴ While disease-causing variants and common sequence variants in some genes have been used to test the accuracy of *in silico* prediction methods,⁵⁻⁹ this has not been done for RYR1. We used PolyPhen-2, PANTHER, PhD-SNP, Pmut, SIFT, MutPred, SNPs&GO and CADD to assess the possibility of using these online prediction tools as a guide to determine whether a variant is neutral, or has the potential to cause MH. We compared the performance of the bioinformatic tools by using the MH-causative sequence variants in RYR1 and CACNA1S, variants of unknown significance in RYR1 and CACNA1S, where functional assays have been performed, and common sequence variants in RYR1 and CACNA1S.

Methods

We compiled data from the literature of MH-causing variants in RYR1 and CACNA1S, and variants that have been associated with MH and have been tested either in a recombinant system or in patient cells for abnormal calcium release, but have not yet been included in the list of causative mutations.

We used SIFT (<http://sift.jcvi.org>), Polyphen-2 (<http://genetics.bwh.harvard.edu/pph2/>), Pmut (<http://mmb.pcb.ub.es/PMut/>), MutPred (<http://mutpred.mutdb.org/>), SNPs&GO (<http://snps-and-go.biocomp.unibo.it/snps-and-go/>) (<http://snps.biofold.org/snps-and-go/snps-and-go.html>), PANTHER (<http://www.pantherdb.org/tools/csnpscoreForm.jsp>), PhD-SNP (<http://snps.biofold.org/phd-snp/phd-snp.html>) and CADD (<http://cadd.gs.washington.edu/home>) to predict the impact of amino acid changes on protein function (all last accessed December 2015). We also investigated the occurrence of the sequence variants in the general population by accessing data from the Exome Variant Server [EVS, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA, (<http://evs.gs.washington.edu/EVS/>), last accessed November 2015] and the 1000 genome project [(<http://www.1000genomes.org/>), last accessed November 2015]. Both the 1000 genome project and EVS combined provide annotated DNA sequence information

for more than 9000 individuals and information about the minor allele frequency (MAF) of variants. We selected common sequence variants according to their occurrence of 1% or above in the 1000 genome project and/or EVS.

SIFT (sorting intolerant from tolerant) predicts the impact of an amino acid change on protein function by comparing amino acid alignments from related sequences to calculate a 'SIFT score': 0-0.05 will be classified as 'damaging', 0.05-1 as 'tolerated'.¹⁰

PolyPhen-2 (Polymorphism Phenotyping vs.-2) uses sequence alignments and phylogenetic and structural data to characterize amino acid substitutions and calculates a score for the variant, classifying it as 'benign', 'possibly damaging' or 'probably damaging'.¹¹ The score ranges from 0.0 (benign) to 1.0 (probably damaging).

Pmut (Prediction of Pathological Mutations on proteins) uses sequence information for its neural network to predict the effect of amino acid changes on protein function, by calculating a reliability index ranging from 0 to 10 (most unreliable to most reliable prediction) and a prediction of either 'neutral' or 'pathological'.¹²

MutPred (Mutation Prediction) is based upon SIFT and structural and functional properties of proteins. MutPred was trained using disease-associated mutations from HGMD and neutral amino acid substitutions from Swiss-Prot. The output contains a general score, (g) where $g > 0.5$ ($P < 0.05$) is actionable, $g > 0.75$ ($P < 0.05$) is confident and $g > 0.75$ ($P < 0.01$) is very confident that an amino acid substitution is likely to have a phenotypic effect.¹³

SNPs&GO uses sequence alignments, 3D protein structure and functional information to calculate if a VUS is neutral or disease-related. The RI score is the reliability index of the predictions, with zero being the most unreliable and ten the most reliable prediction.¹⁴

PANTHER (Protein Analysis Through Evolutionary Relationships) groups proteins according to their 'evolutionary relationships, molecular function, biological processes and function' and classes the variants as either 'neutral' or associated with 'disease', assigning every variant a reliability index (RI) ranging from zero to 10 (most unreliable to most reliable prediction). The alignments are based on hidden Markov models (HMM).¹⁵

PhD-SNP (Predictor of Human Deleterious SNP) uses support vector machine prediction trained on sequence and evolutionary information to classify variants as 'neutral' or 'disease' causing. The RI (Reliability Index) of the predictions ranges from zero to 10 (most unreliable to most reliable prediction).¹⁶

CADD (Combined Annotation Dependent Depletion) combines a wide range of annotations including functional genomic data (e.g. transcription factor binding), transcript information, conservation and protein level scores (including SIFT and PolyPhen).¹⁷ CADD provides a 'raw' C-score and a 'scaled' PHRED-like scaled c-score [$(-10 * \log_{10}(\text{rank}/\text{total}))$]. A scaled PHRED-like score of 10 indicates that a variant is predicted to be in the 10% of most deleterious substitutions possible in the human genome; a score of 20 is between the 1% most deleterious. The suggested cut-off by the authors is between 10 and 20.

We calculated the probability of identifying true deleterious variants as the sensitivity and the specificity as the probability of identifying true neutral variants. Sensitivity=[true positives/(true positives+false negative)], specificity=[true negatives/(true negatives+false positives)].

Results

MH-causative mutations

MutPred, SNPs&GO, PhD-SNP and CADD (cut-off 15) predicted all MH-causative variants in RYR1 (G248R is listed twice, RYR1

c.742G>A and c.742G>C, (see Supplementary data, file and Table 1) to be associated with disease. SIFT identified three as being tolerated, Pmut identified five as neutral and PolyPhen-2 identified one as benign. PANTHER predicted all but two of the variants to be associated with disease because of failure to align to HMM. I403M (c.1209C>G) is designated neutral and tolerated by Pmut and SIFT respectively. Only three of the MH-causative variants are listed in the EVS: R530H [c.1589G>A, minor allele frequency (MAF) 0.000154], R614C (c.1840C>T) and A2350T (c.7048G>A, both MAF 0.00008). Of the two MH-causative variants in CACNA1S (Supplementary data, file), PolyPhen-2, SIFT, MutPred, SNPs&GO, PANTHER, PhD-SNP and CADD (cut-off 15) all associated both variants with disease, whereas Pmut predicted the R1086H (c.3257G>A) variant to be neutral. Neither of the two causative CACNA1S variants is listed in EVS.

VUS tested in calcium release experiments

Each of the VUS in RYR1 in Supplementary data, file and Table 3 have been demonstrated to either result in abnormal calcium release from the SR (dyspedic myotubes) or endoplasmic reticulum (HEK 293 cells) or lead to altered resting calcium concentrations.

Four of the variants (R533H, c.1598G>A; D4505H, c.13513G>C; F4808L, c.14424C>A; and A4906V, c.14717C>T) were predicted to have no impact on protein function by at least one prediction programme. Interestingly, two out of the four variants (R533H, c.1598G>A; D4505H, c.13513G>C) are found in EVS with an allele count of seven of 12999 (MAF 0.00054) and 43 of 12955 (MAF 0.003) respectively. MutPred and PhD-SNP called the D4505H (c.13513G>C) variant neutral, SNPs&GO and PANTHER called the F4808L (c.14424C>A, CCD) variant neutral, SIFT called the A4906V (c.14717C>T) variant neutral (Table 1).

One variant in CACNA1S (T1354S, c.4060A>T) predicted to be 'neutral' by seven out of the eight programmes tested (only MutPred predicted that the variant would have an impact on protein function) had previously been shown to lead to increased RyR1 calcium release in dysgenic mouse cells and had an allele count of 48 of 12958 in EVS (MAF 0.0037, Supplementary data, file).¹⁸

Lymphoblastoid cell lines generated from patient blood samples had previously been used to demonstrate that the RYR1 VUS (shown in the Supplementary data, file) resulted in abnormal

calcium release. Seven variants were predicted to have no impact on protein function by at least one prediction programme (Table 1). Surprisingly two of the variants in RYR1 (E1058K, c.3172G>A and K1393R, c.4178A>G) were identified as 'benign' by five and six respectively of the eight prediction programmes used. One of these variants (K1393R, c.4178A>G) was found in EVS with an allele count of 56 of 12942 (MAF 0.0043).

Of the seven variants analysed in myotubes derived from patients (Supplementary data, file), I2182F (c.6544A>T) was predicted as 'neutral' by Pmut and PANTHER, whereas R3348C (c.10042C>T) was predicted to be 'benign' by MutPred (Table 1), I2453T was predicted to be benign by PolyPhen-2. All other variants were predicted as having an impact on protein function.

Common sequence variants

The common sequence variants were selected according to their MAF in the 1000 Genomes Project and EVS databases (Supplementary data, file) and two of the RYR1 variants (G2060C, c.6178G>T and P1787L, c.5360C>T) had previously been tested in HEK293 cells.^{19,20} Panther failed to predict four of the six variants because the variant position did not align to an HMM. Only three of the six common sequence variants in RYR1 were classed as benign by all the other seven prediction programmes (Table 2).

All common sequence variants in CACNA1S selected for the analysis (Supplementary data, file) were predicted to be associated with disease, by at least one of the programmes tested (Table 2). SNPs&GO and CADD predicted all common sequence variants in CACNA1S as associated with 'disease'; PhD-SNP classed one as benign.

Discussion

The advent of targeted DNA capture and next generation sequencing (and the associated reductions in the cost of DNA sequencing) has led to more research and clinical groups using NGS to identify disease-causing variants. One of the bottlenecks in the data analysis however is the sheer number of variants that are identified in each individual. Common sequence variants can be filtered out according to their minor allele frequency, but all other rare variants need to be investigated. One of the questions is where to set the threshold for filtering out 'common' variants to leave only the potential causative variants. The frequency of MH-causative variants in EVS is <0.001. If the threshold is set too high, there are too many variants to consider, but if it is set

Table 1 Performance of prediction tools for variants of unknown significance in RYR1 shown to lead to abnormal calcium release, or altered resting calcium concentrations in a recombinant system and in patient cells. ^A cut-off 15

	Sensitivity HEK293 cells or dyspedic myotubes (n=20)	Sensitivity Lymphoblastoid cell lines (n=11)	Sensitivity Muscle cells from patients (n=7)
Polyphen-2	1	0.64	0.86
Pmut	1	0.64	0.86
SIFT	0.9	0.64	1
MutPred	0.95	0.73	0.86
SNPs&GO	0.95	1	1
PANTHER	0.95	0.55	0.71
PhD-SNP	0.95	0.82	1
CADD ^A	1	1	1

Table 2 Performance of prediction tools for the known MH-causing variants and common sequence variants in RYR1 and CACNA1S. ^A cut-off 15

	Sensitivity RYR1 (n=35)	Specificity RYR1 (n=6)	Sensitivity CACNA1S (n=2)	Specificity CACNA1S (n=6)
Polyphen-2	0.97	1	1	0.33
Pmut	0.86	0.67	0.5	0.67
SIFT	0.91	1	1	0.67
MutPred	1	0.83	1	0.83
SNPs&GO	1	0.83	1	0
PANTHER	0.94	0	1	0.5
PhD-SNP	1	1	1	0.17
CADD ^A	1	0.5	1	0

too low, potential disease causing variants might be filtered out. The 1000 Genomes Project (<http://www.1000genomes.org/>) and EVS (<http://evs.gs.washington.edu/EVS/>) not only display the minor allele frequency of variants, they also show the bioinformatic prediction by SIFT, PolyPhen and PolyPhen-2. The use of bioinformatic tools combined with segregation studies and MAF can help researchers to determine which variants might be potentially disease-causing. These bioinformatic tools are designed to discriminate between neutral and pathogenic variants. Amino acid substitutions in a protein might disrupt binding sites for other proteins, catalytic residues and protein folding (structure), however not all amino acid substitutions result in altered protein function.

All of the bioinformatic programmes used in this study are available online. We focused on eight pathogenicity prediction methods, although there are many more available. Some of the tools allow batch queries, which make high-throughput analysis easier. The outputs of the analysis can be either in numerical (showing the probability of the missense variant resulting in compromised protein function) or textual (benign, neutral, tolerated, probably damaging, possibly damaging, damaging, pathological, disease) form. Some of the programmes (Pmut, SNPs&GO), give two possible results (neutral, pathological/disease-causing) with associated probability, while others have three possible outcomes (e.g. PolyPhen-2 benign, possibly damaging, probably damaging). For CADD, the researchers have to define their own cut-off point which could be different for different genes. Depending on which web server is used (some of the prediction tools can be accessed through different host servers), the outcome might vary slightly, which is an important detail to consider. In addition, PANTHER did not give a prediction for some of the variants because it failed to align to an HMM.

The majority of MH-causative sequence variants were predicted correctly by the different bioinformatic programmes. Pmut failed to predict six MH-causative variants correctly and therefore performed the worst. MutPred, SNPs&GO, PhD-SNP and CADD predicted all MH-causative variants correctly.

The common sequence variants were selected according to their MAF in EVS and 1000 genomes and some variants with lower MAF were excluded, as they have not been proved to be neutral using functional assays. Correct prediction of the common sequence variants in general was lower compared with the MH-causative variants, however the total number of variants analysed was also lower.

The recommended cut-off for CADD is between 10 and 20; we chose 15 which worked well for the MH-causative variants. Setting the cut-off at 20 would have improved the correct predictions of common sequence variants in *CACNA1S* only slightly (0.17 instead of zero), but would not have changed the sensitivity of the predictions. A cut-off above 22 would have affected the sensitivity of the predictions and produced false negative results.

Different research groups use different systems to determine if a VUS alters calcium release or not. Currently, the most direct measurement of abnormal calcium release caused by a variant in *RYR1* is to express recombinant mutated *RyR1* in either HEK293 cells or dyspedic myotubes. Measurement of calcium release using myotubes or B-lymphoblastoid cell lines derived from patients will not exclude the patient's genetic background and therefore can only be used as an indirect measure, as the patients might have additional variants in a gene or genes expressed in muscle tissue or lymphocytes that may act as modifiers. While the recombinant systems are not affected by this, their results must still be treated with some caution. Transfection of HEK293 cells with mutated *RYR1* cDNA results in homozygous expression

of the variant in question, whereas the patient is very often heterozygous for the variant. Mutated and wildtype *RYR1* cDNA can be co-transfected at the same time to achieve heterozygous expression, but how the tetrameric calcium channel is assembled with mutated or wildtype subunits cannot be accurately predicted.

The programmes performed very well for variants that had been tested in a recombinant system and in patients' muscle cells, but were not as accurate for variants tested in lymphoblastoid cell lines. This might be a reflection of the patient's genetic background interfering when calcium release assays are performed in lymphoblastoid cell lines. Alternatively it may be a result of the smaller calcium stores in lymphocyte endoplasmic reticulum, hence sensitivity for the assay is not as high as in myotubes or HEK293 cells. Lymphocytes do not express other proteins known to be part of the skeletal muscle triad and can therefore only be used to assay calcium release from *RyR1*. On the other hand, lymphocytes only require a blood sample from the patient, not a biopsy, they can be easily grown, are easy to handle and assay and therefore offer a system to assay calcium release when there are no muscle cells available, or for a first screen before cloning the variants and using a recombinant system.

None of the programmes predicted all the MH-causative sequence variants, VUS and common variants correctly. Overall MutPred, SNPs&GO, PhD-SNP and CADD performed with the highest sensitivity (true positives), SIFT and MutPred had the highest specificity (true negatives).

At the moment, variants of unknown significance are classified as pathogenic, likely pathogenic, uncertain, likely not pathogenic and not pathogenic²¹ according to all the available information including family segregation with disease, MAF (EVS, 1000 genomes), position in protein, functional assays and bioinformatic pathogenicity prediction. Access to databases such as HGMD may further assist the classification of VUS as pathogenic or benign.²²

While bioinformatics tools may be useful in identifying variants for functional analysis, even the use of at least three programmes in combination as suggested by Wallis and colleagues 2013²³ should be treated with caution. We strongly recommend use of the results of bioinformatic analysis only in combination with other available data such as IVCT results, family segregation analysis, functional analysis, literature and MAF. At this point we and other researchers do not believe that the available tools are accurate enough to give a definitive answer and predictions should be treated with caution.^{8 9 24}

Authors' contributions

Study design/planning: A.H.S.

Study conduct: A.H.S.

Data analysis: A.H.S., K.M.S.

Writing paper: A.H.S., K.M.S.

Revising paper: all authors

Supplementary material

Supplementary material is available at *British Journal of Anaesthesia* online.

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Declaration of interest

None declared.

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