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Assessing the pathogenicity of *RYR1* variants in malignant hyperthermia

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Abstract

Background: Missense variants in the ryanodine receptor 1 gene (*RYR1*) are associated with malignant hyperthermia but only a minority of these have met criteria for use in predictive DNA diagnosis. We examined the utility of a simplified method of segregation analysis and a functional assay for determining the pathogenicity of recurrent *RYR1* variants associated with malignant hyperthermia.

Methods: We identified previously uncharacterised *RYR1* variants found in 4 or more malignant hyperthermia families and conducted simplified segregation analyses. An efficient cloning and mutagenesis strategy was used to express ryanodine receptor protein containing one of six *RYR1* variants in HEK293 cells. Caffeine-induced calcium release, measured using a fluorescent calcium indicator, was compared in cells expressing each variant to that in cells expressing wild type ryanodine receptor protein.

Results: We identified 43 malignant hyperthermia families carrying one of the six *RYR1* variants. There was segregation of genotype with the malignant hyperthermia susceptibility phenotype in families carrying the p.E3104K and p.D3986E variants but the number of informative meioses limited the statistical significance of the associations. HEK293 functional assays demonstrated an increased sensitivity of RyR1 channels containing the p.R2336H, p.R2355W, p.E3104K, p.G3990V and p.V4849I compared to wild type but cells expressing p.D3986E had a similar caffeine sensitivity to cells expressing wild type RyR1.

Conclusions: Segregation analysis is of limited value in assessing pathogenicity of *RYR1* variants in malignant hyperthermia. Functional analyses in HEK293 cells provided evidence to support the use of p.R2336H, p.R2355W, p.E3104K, p.G3990V and p.V4849I for diagnostic purposes but not p.D3986E.

Keywords

Malignant hyperthermia; genetics, diagnosis, pathophysiology; HEK293; calcium regulation; *RYR1*

Malignant hyperthermia (MH) is a rare, pharmacogenetic disorder triggered in susceptible individuals upon exposure to volatile anaesthetics and depolarizing neuromuscular blocking drugs[1]. Upon triggering, patients suffer a hypermetabolic reaction caused by failure to regulate calcium homeostasis in skeletal muscle. The type I ryanodine receptor gene (*RYR1*) is the primary locus for MH with 189 variants in the coding sequence identified in association with the disease (www.emhg.org). Despite this, MH is a heterogeneous disorder with several other loci implicated: mutations have been identified in *CACNA1S* [2-4], which encodes the alpha 1 subunit of the dihydropyridine receptor, $Ca_v1.1$. Most recently homozygosity for p. W284S in *STAC3* has been implicated in Native American myopathy, which is associated with MH [5]. There remains a significant minority of cases, however, in which no mutation is found in *RYR1*, *CACNA1S* or *STAC3*. Our group and others are using next generation sequencing to identify variants in these genes and novel genes potentially implicated in susceptibility to MH [6-9] and this is generating a large number of variants of uncertain significance. The European Malignant Hyperthermia Group (EMHG) established a set of criteria that variants associated with MH should meet before they can be included onto the genetic diagnostic panel for MH [10]. If patients are found to possess a variant meeting these criteria, they can be diagnosed at high risk of developing MH under anaesthesia. If no such variant is found, diagnosis requires a muscle biopsy and subsequent *in vitro* contracture tests (IVCT) for diagnosis [10]. To comply with the EMHG guidelines, variants have to be functionally characterized. For *RYR1* variants, the coding region of 15 kb makes the cloning of full length *RYR1* mutant constructs and subsequent transduction of muscle cell lines technically challenging, time-consuming and costly. None of the published experimental models for functional analysis of *RYR1* variants is ideal but the most frequently used is heterologous expression in HEK293 cells [11-13]. This system is not ideal because

RyR1 normally functions in the architecturally complex environment of skeletal muscle and in the presence of numerous accessory proteins not expressed in other cell types, including HEK293 cells. In the absence of an ideal system for the functional characterization of *RYR1* variants, the expert consensus of the EMHG, in formulating its guidelines,[10] was to accept pragmatically the use of heterologous expression in HEK293 cells along with other imperfect models.

A consequence of the constraints on functional analysis of *RYR1* variants is that only 34 variants have been added to the diagnostic panel out of 189 reported to be associated with MH susceptibility (www.emhg.org). Urwyler and colleagues [14] proposed the use of linkage analysis as an alternative means of assessing likely pathogenicity. The requirement for a specialized invasive procedure (muscle biopsy and IVCT) for accurate phenotyping and the lack of validity in combining families for linkage in a heterogeneous disorder has meant this is not a realistic option. Møller and colleagues [15], however, proposed a simplified method for segregation analysis (SISA) where combining the number of informative meioses from more than one family carrying the same variant can legitimately be used to generate a probability of association between genotype and phenotype. Use of the SISA method has been proposed as a tool to identify likely pathogenic variants in generic guidelines for classification of genetic variants [16].

The aims of this study are to assess the utility of SISA and functional characterization in HEK293 cells for determining the likely pathogenicity of *RYR1* variants found in 43 families from the UK MH population with a sub aim to determine if there was equipoise between different methods of transfecting the HEK293 cells. If these variants were confirmed to be at

least likely to be pathogenic, their adoption onto the diagnostic panel for MH would increase by up to 14% the number of the >770 families in the UK where DNA diagnostics would be applicable.

Methods

Variant selection and identification of families carrying variants

We selected *RYR1* variants that we had found in 4 or more families in association with MH susceptibility but which had not been functionally characterized. We began screening our patient resource for *RYR1* variants following publication of the first *RYR1* variant implicated in MH susceptibility [17]. As further *RYR1* variants were implicated our screening programme evolved into a systematic search for all published variants principally using amplification refractory mutation system or restriction digest assays. From the early 2000s it became economically feasible to use traditional (Sanger) sequencing of mutation "hot-spots" and even the whole coding region of *RYR1* and *CACNA1S* [18]. Most recently, next generation sequencing (NGS) technology has been employed. We have screened members of 652 independent MH families for the presence of the variants of interest, using one or more of amplification refractory mutation system or restriction endonuclease digest assays, Sanger sequencing or NGS.

Segregation analyses

For each family carrying one of the variants of interest, the number of informative meioses was calculated as one less (to account for ascertainment bias) than the number of affected carriers (including obligate carriers). The number of informative meioses for all families carrying a single variant were summed before calculating the probability that the variant is

not disease causing according to Møller and colleagues [15]. British/Dutch guidelines [16] have suggested that the presence of genotype-phenotype discordance (affected non-carrier or non-affected carrier) precludes the use of the SISA method of predicting disease association. When we encountered cases of discordance, we reviewed the IVCT records (phenotype) and calculated the probability that the IVCT responses represented an abnormal response [19]. We also verified the genotype using Sanger sequencing where a DNA sample was available and, again when feasible, used deep resequencing of *RYR1* and *CACNA1S* to look for alternative disease-associated variants in cases of affected non-carriers.

Cloning

Human *RYR1* cDNA was inserted into a pcDNA3.1 expression vector (containing a neomycin resistance gene cassette) to produce pc*RYR1* (20,649bp in size) as reported previously [12]. Sequences were aligned to the *RYR1* reference sequence NM_000540.2 with complementary DNA (cDNA) positions 1, 2 and 3 defined as the ATG start codon. pc*RYR1* was split into two roughly equal subclones containing the entire *RYR1* coding sequence between them. The 5' subclone spanned from a *SpeI* site within the pcDNA 3.1 backbone to the *HindIII* site at cDNA position 9553. The 3' subclone spanned from the *Acc65I* site at cDNA position 6,980 to an *XbaI* site in the pcDNA 3.1 backbone. Both fragments were inserted into a pBluescript SK II + vector to create subclones of 12.523bp and 11,128bp, respectively.

These subclones were used for mega primer polymerase chain reaction (PCR) of whole plasmid (MEGAWHOP) site-directed mutagenesis as reported previously [20,12]. Briefly, a

forward mutagenic primer was paired with a wild type reverse primer to create mega primers between 200 bp and 800 bp in length containing the variant. The mega primers were sequenced to confirm the presence of the mutation before using them for whole plasmid mutagenesis of one of the subclones using the QuikChange protocol (Agilent Technologies, Wokingham, UK). Mutant subclones were sequenced to confirm successful mutagenesis.

pcDNA

The p.R2336H, p.E3104K, p.D3986E, p.G3990V and p.V4849I variants in the 3' subclone were reconstructed into the pc*RYR1* plasmid by using the same *Acc65I* and *XbaI* sites used to create the subclone.

pTUNE

Wild type, p.R2355W and p.D3986E *RYR1* constructs were inserted into a pTUNE inducible expression vector [21] using both subclones. Initially, an endogenous *BspEI* restriction site had to be removed from the pTUNE neomycin resistance gene by digesting with *BspEI* and blunting with DNA polymerase I (Klenow fragment). The entire 3' end of *RYR1* was removed from wild type, p.R2355W and p.D3986E 3' subclone constructs using the same *Acc65I* restriction site that was used for creating the subclone paired with a *SalI* site downstream of the stop codon of *RYR1*. This fragment was inserted into the modified pTUNE vector by ligation into compatible *BsiWI* and *XhoI* restriction sites. The wild type 5' subclone fragment was removed using the same *SpeI* restriction site used to create the subclone and the *BspEI* site at cDNA position 7,073. The 5' fragment was inserted into the pTUNE subclone using a compatible *NheI* site for the *SpeI* site and the *BspEI* site that was intact in the 3' end of *RYR1* to create full-length pTUNE*RYR1* (pt*RYR1*) constructs 27,279bp in size.

Cell culture and transfection

HEK293 cells were maintained according to the supplier's (LGC Standards, Teddington, UK) recommended conditions (Dulbecco's Modified Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Biosera, Uckfield, UK) and 100 units/mL of penicillin and 100 µg/mL of streptomycin and cultured at 37°C and 5% CO_2 in a humidified incubator).
Stable transfection of HEK293 cells was performed in 6-well plates. Transfections were performed using Lipofectamine-2000 according to manufacturer's instructions. Initial selection pressure was performed using 1 mg/mL G418 for 10 days before lowering the antibiotic concentration to 400 µL/mL through several rounds of selection until individual colonies were selected as stable clones. Transfected cells were seeded onto entactin-collagen IV-laminin (ECL) coated 96-well Opti-Clear plates (Corning Incorporated) 24 h prior to calcium measurements.

Transient pc*RYR1* and pTUNE*RYR1* transfections were performed on ECL coated 96-well Opti-Clear plates that were seeded with HEK293 cells 24 h prior to transfection as described above. For pc*RYR1* transfections, the medium was changed 6 h after transfection and incubated for 48 h prior to calcium imaging. For pt*RYR1* transfections, 6 h post-transfection, the medium was changed to one containing 25 µM isopropyl-β-thiogalactopyranoside (IPTG) to induce a consistent level of RyR1 expression. Fresh medium containing IPTG was added every 24 h and caffeine-induced calcium release experiments were performed 72 h post-transfection.

Western blotting

Transfected and untransfected HEK293 cells were lysed with 100 mM Tris and 0.5% (v/v) Triton X-100. The supernatant from the cell lysis procedure was isolated and quantified using a Bradford assay (Bio-Rad). Fifty µg of whole protein was run on a 4%-8% polyacrylamide gel at 100 volts for 2 h. After electrophoresis, the separated protein was transferred to a polyvinylidene fluoride (PVDF) membrane overnight at 4°C. RyR1 protein was detected using a 34C mouse anti-RyR1 primary antibody. The primary antibody was detected using a goat-anti-mouse secondary antibody with a horseradish peroxidase (HRP) conjugate. Chemiluminescence was detected using the North2South HRP detection kit (Thermo Scientific).

Imaging

Prior to imaging, transfected cells were washed four times in a Ringer's solution (125 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 6 mM glucose, 25 mM HEPES, pH 7.4) and incubated with 20 µM Fluo-4 AM in the dark for 20 min at room temperature (Life technologies). Excess Fluo-4 was washed off an additional four times with Ringer's solution. Caffeine-induced calcium release experiments were performed using the system described by Duke and Steele [22]. Briefly, a caffeine dilution series was created in Ringer's solution. Experiments on stable HEK293 cell lines were carried out using 0.5 mM, 1 mM, 2 mM, 5 mM and 20 mM caffeine. Experiments on transiently transfected HEK293 cells with the p.G3990V construct were performed using 0.2 mM, 0.5 mM, 1 mM, 2 mM, 5 mM, 10 mM and 20 mM caffeine. The remaining transient transfection experiments and all experiments involving pt*RYR1* constructs were performed using a simplified series of 0.5 mM, 1 mM, 2 mM, 4 mM, 8 mM and 20 mM caffeine. In all experiments a wild type and untransfected (data not shown) control was included. Additionally, control experiments were performed to

ensure that the difference in caffeine series did not significantly alter the EC_{50} calculated from the data (data not shown). Each caffeine solution was loaded into a syringe and the syringes containing solutions of different caffeine concentration were attached to a central column. Each syringe was under computer control and sequentially injected caffeine at standardized levels from the lowest concentration to the highest. Each dose of caffeine was perfused off the cells before the next dose to prevent additional calcium release events due to remaining caffeine. Caffeine-evoked calcium release was observed using confocal microscopy as a temporary increase in fluorescence directly after the application of caffeine.

Data handling and analyses

Simplified segregation analyses were carried out according to Møller and colleagues [15].

In the functional studies, only cells that responded to concentrations of caffeine < 4 mM were selected for analysis as a small proportion (average 4%) of untransfected cells responded to higher (8 mM-20 mM) concentrations of caffeine. Videos were imported into ImageJ software, responding cells were selected as regions of interest and their fluorescence levels over time were plotted using the Prism 6 statistics package. Each video trace was individually normalized with 0 % defined as the level of fluorescence before the addition of any caffeine and 100 % defined as the peak fluorescence obtained at that concentration. Normalized traces were used to construct concentration-response curves by plotting the peak fluorescence generated at each caffeine concentration on a logarithmic scale before using the non-linear regression function in Prism 6 to create the curve. The concentration at which half of the maximum response was generated (EC_{50}) was calculated from the concentration-response curves. Data are presented as mean, standard error (SE) and 95%

confidence intervals (95% CI). Statistical inference was based on unpaired Student's *t*-tests, using a *P* value < 0.05 as evidence of statistical significance.

Results

RYR1 variants

The following *RYR1* variants (n=number of families) met our criteria for inclusion in this study: c.7007G>A, p.R2336H (n=8); c.7063C>T, p.R2355W (n=7); c.9310G>A, p.E3104K (n=4); c.11958C>G, p.D3986E (n=5); c.11969G>T, p.G3990V (n=11); c.14545G>A, p.V4849I (n=8). The evolutionary conservation of the nucleotide and encoded amino acids between species and RyR isoforms are shown in supplementary tables 1 and 2 and the physicochemical consequences of the amino acid substitutions, and the minor allele frequencies of the variants are shown in supplementary table 3.

Segregation analyses

Table 1 shows the number of families in which the familial variant either segregates completely with the MH susceptible phenotype (determined by the *in vitro* contracture tests [10]) or not. Also presented are the total number of informative meioses in those families where there is segregation and, in those families where there is no genotype-phenotype discordance, the probability of segregation occurring by chance, both of which were calculated according to the SISA method [15].

There were 8 affected non-carriers in 7 families but no non-affected carriers. The pedigrees of these families are shown in supplementary figure 1. Analyses of the *in vitro* contracture responses [19] of the affected non-carriers gave a predicted probability of true susceptibility to

MH of > 0.9 in all cases (data not shown). The absence of the familial variant was confirmed in the affected non-carriers by sequencing stored DNA in all but one case, where DNA was not available (individual IVii in family 1, supplementary figure 1). Sufficient high quality DNA was available for deep resequencing of the entire coding sequence of *RYR1* and *CACNA1S* in 3 affected non-carriers, one of whom (individual IIIii in family 5, supplementary figure 1) was found to harbour a rare *RYR1* variant (c.6478G>A, p.G2160S).

Functional studies

Ca^{2+} Imaging in stably transfected HEK293 cells using pc*RYR1* constructs

Stable HEK293 cell lines were produced for wild type *RYR1* and 3 of our 6 *RYR1* variants (arbitrarily chosen): p.R2336H, p.D3986E and p.G3990V. Stable cell lines were exposed to 0.5 mM, 1 mM, 2 mM, 5 mM and 20 mM caffeine and the fluorescence data over time were plotted (Figure 1b). Concentration-response curves for each variant are illustrated in Figure 1c-e and the EC_{50} values plotted in Figure 1f. For cells transfected with p.R2336H and p.G3990V constructs, the fitted curve was shifted to the left, indicating an increase in sensitivity to caffeine. Compared to cells stably transfected with the wild type pc*RYR1* construct where the mean EC_{50} was 3.21 mM (95% CI 2.44-3.77 mM), p.R2336H and p.G3990V transfected cells had EC_{50} values that were statistically significantly reduced to 0.91 mM (95% CI 0.80-1.27 mM; *P*=0.0003) and 1.41 mM (95% CI 1.12-1.69 mM; *P*=0.0001) respectively. The EC_{50} of cells transfected with the p.D3986E construct was 2.86 mM (95% CI 2.23-3.48 mM), which was not statistically significantly different to wild type (P=0.23).

Ca^{2+} Imaging in transiently transfected HEK293 cells using pc*RYR1* constructs

Transient transfection of HEK293 cells was performed using the wild type, p.E3104K and p.V4849I constructs (arbitrarily chosen over p.R2355W) and the p.G3990V construct, which was selected to make a comparison between stable and transient transfection. Representative fluorescent traces produced from each construct are presented in Figure 2b. For each of the *RYR1* variants, the caffeine dose-response curve was shifted to the left indicating an increase in sensitivity to caffeine (Figure 2c-e). HEK293 cells transiently transfected with each of the three variants produced a statistically significant decrease in EC_{50} response to caffeine as compared to wild type (EC_{50} for wild type, 4.1 mM, 95% CI 1.64-5.95): p.E3104K had an EC_{50} of 1.74 mM (95% CI 1.2-2.62; *P*=0.01 compared to wild type, n=7); p.G3990V had an EC_{50} of 1.25 mM (95% CI 0.76-1.62; *P*=0.01, n=6); p.V4849I had an EC_{50} of 1.71 mM (95% CI = 1.3-2.21; *P*=0.03, n=7).

Ca^{2+} Imaging in transiently transfected HEK293 cells using pt*RYR1* constructs

By the time we were in a position to start work on the remaining *RYR1* variant to be characterized (p.R2355W), we had demonstrated that transient transfection produced similar results as stable transfection for p.G3990V, while cells expressing p.D3986E after stable transfection produced similar caffeine responses to cells expressing wild type RyR1. In order to exclude the possibility that the findings with p.D3986E were a consequence of variable expression we progressed to the pTUNE system for transient transfection in order to regulate *RYR1* expression. HEK293 cells were transfected with wild type, p.R2355W and p.D3986E pt*RYR1* variants. Expression of *RYR1* was induced using IPTG at a standard concentration of 25 μM. Seventy-two hours post-transfection, cells were loaded with Fluo-4 AM and exposed to incremental doses of caffeine. Uninduced and untransfected cells were

indistinguishable from each other (data not shown) but cells that were transfected always responded in a concentration-dependent manner after induction. Representative traces for the wild type, p.R2355W and p.D3986E constructs are shown in Figure 3b. Concentration-response curves (Figure 3c-d) illustrate the increased sensitivity of p.R2355W to caffeine (EC_{50} of 1.38 mM, 95% CI 0.02-2.3 mM) compared with wild type (3.8 mM, 95% CI 2.52-5.4; *P*=0.048, n=4) but p.D3986E showed similar sensitivity to caffeine (EC50 4.67 mM, 95% CI 3.4-5.7 mM) as wild type (*P*=0.69).

Discussion

The work presented in this paper highlights several issues when attempting to classify *RYR1* variants for their potential pathogenicity in relation to malignant hyperthermia susceptibility. We have the most extensive phenotypically characterized MH patient resource in the world, but we found that segregation analysis using a method that enables data from multiple families to be simply combined was of limited value in defining the pathogenicity of *RYR1* variants. For the two variants that segregated with the MH susceptibility phenotype (p.E3104K, p.D3986E), the limited number of informative meioses precludes definitive conclusions for a condition demonstrating locus heterogeneity, where a SISA significance level of $P < 0.001$ would be analogous to a LOD score of >3 using linkage analysis. For the remaining variants, the incidence of genotype-phenotype discordance limits the use of SISA analysis.

Our functional studies of recombinant RyR1 in the heterologous HEK293 system suggest, however, that the p.R2336H, p.R2355W, p.E3104K, p.G3990V and p.V4849I *RYR1* variants produce a RyR1 protein with increased sensitivity to the agonist caffeine. Such gain of

function effects are consistent with a pathogenic role in malignant hyperthermia. On this basis, these variants meet the criteria of the European MH Group [10] for predictive diagnostic DNA screening as high-risk variants. These five variants have been identified in a total of 38 families in the UK and their addition to the diagnostic panel of functionally characterized *RYR1* and *CACNA1S* variants would increase the availability of diagnostic DNA screening in the UK MH population by 14%. These variants have been reported in at least a further 13 families worldwide.

The p.R2336H, p.R2355W and p.V4849I variants have been identified in other populations and previous attempts have been made to assess their functional consequences using primary cells (lymphoblastoid cells or skeletal myoblasts) derived from patients carrying the variant. Although this type of experimental model is included, with proviso, in the EMHG guidelines for functional analysis of *RYR1* variants[10], its use is controversial. As with recombinant HEK293 cells, expression of all the components of the excitation-contraction of adult skeletal muscle is not recapitulated in lymphoblastoid cells but, additionally, even in myotubes derived from patient skeletal myoblasts, the effects of the *RYR1* variant under investigation cannot be isolated from those of other potentially pathogenic variants in the patient's genetic background.

Lymphoblastoid cells obtained from patients carrying the p.R2336H variant only produced an MH phenotype when exposed to 4-chloro-*m*-cresol (4-CmC). The response obtained from these cells when they were exposed to caffeine was similar to that of wild type familial control samples [23]. The reason for the apparent wild type response to caffeine in this system is not clear, especially given the positive result obtained using 4-C*m*C. Additionally, the 6

families in the UK that possess this variant produced a typically strong reaction to caffeine during IVCT MH diagnosis [24]. The functional data presented in the recombinant system used in this study, however, provides clear evidence of an increased sensitivity to caffeine in cells expressing these variants. The data may suggest that the lymphoblastoid system [23] is unsuitable for functional experiments.

Ducreux and colleagues [25] presented functional data from lymphoblastoid cells carrying the p.V4849I variant that showed responses to both caffeine and 4-C*m*C which were indistinguishable from wild type cells. Originally this result was attributed to the fact that the p.V4849I variant has also been identified in association with central core disease (CCD), a congenital myopathy also associated with *RYR1* mutations that has been known to result in reduced calcium release from stores due to a higher passive calcium leak [26-28]. Additionally, the p.V4849I variant has been identified in a patient at low risk for MH susceptibility, leading to suggestions that this variant is not a pathogenic MH mutation [29]. We suggest that our data obtained here using recombinant expression in HEK293 provide the clearest evidence to date that the p.V4849I variant has a pathogenic role in MH. No evidence of cores was found on histological examination of the probands' muscle from each of our families found to carry this variant.

The p.R2355W variant has been identified in 11 families worldwide and two studies [30,31] have presented functional data for this variant using primary cells isolated from a total of three families. The results were indicative of a pathogenic MH variant and our data from recombinant expression in HEK293 confirm these findings.

Our results in HEK293 cells expressing the p.D3986E *RYR1* variant were unexpected. Although we had a limited number of informative meioses in the 5 families where this variant is present there was no genotype-phenotype discordance. Analyses of IVCT responses in these families had previously suggested that the variant was associated with a relatively "strong" phenotype [24]. We initially used a pcRYR1 construct to stably transfect HEK293 cells and found no difference in caffeine sensitivity between cells expressing p.D3986E and those expressing wild type RyR1. In order to test the possibility that variable expression of RyR1 p.D3986E in stably transfected cells might influence the caffeine responses, we carried out further experiments where we transiently transfected HEK293 cells using the inducible pTUNE vector to ensure equivalent amounts of RyR1 protein in wild type and mutant cells. Again, the responses of cells expressing p.D3986E and those expressing wild type RyR1 were similar. These functional studies suggest that either the p.D3986E variant does not alter the function of the RyR1 or any such changes require co-expression of proteins normally expressed in skeletal muscle but not HEK293 cells.

We were also interested in exploring whether both stable and transient expression in HEK293 cells produce similar results for assessing the functional significance of *RYR1* variants. Caffeine sensitivity of the p.G3990V was determined using both methods of transfection. The EC_{50} values for 1.41 mM (stable) and 1.25 mM (transient) transfection were similar and both significantly different to the wild type cells transduced using the same method. The EC_{50} values for cells expressing wild type after transient (using either construct) or stable transfection were similar as were those for cells expressing p.D3986E after transient (pTUNE) or stable transfection.

Four of the *RYR1* variants (p.R2336H, p.R2355W, p.G3990V and p.V4849I), which were associated with increased caffeine sensitivity in the HEK293 expression system, were found in families where segregation between the variant and the IVCT phenotype was incomplete (Table 1). Where possible we excluded genotyping errors in discordant individuals and also calculated the likelihood of false positive IVCT responses in each affected non-carrier and conclude that it is extremely unlikely that this is a satisfactory explanation. Similar conclusions have been drawn from previous studies of genotype-phenotype discordance [32]. Our current data provide further evidence that more than one genetic factor is operating in at least a minority of MH families [33,34,24]. Indeed, in one family where the p.V4849I variant was detected initially, in a non-carrier of p.V4849I, who had a positive IVCT, a second rare *RYR1* variant was found when the entire coding region of *RYR1* was sequenced using deep resequencing. Deep resequencing did not detect a second *RYR1* variant in IVCT positive non-carriers from two other families but this does not exclude variants in other, as yet unidentified, genes to be implicated in the MH susceptibility phenotype. Another possibility, which we consider unlikely, is that the *RYR1* variants are not associated with MH susceptibility and the increased caffeine sensitivity in the HEK293 system is not representative of the functional effects of these variants in human skeletal muscle.

In conclusion, we have confirmed that segregation analysis has limited utility in establishing likely pathogenicity of *RYR1* variants associated with MH susceptibility. Our functional data meet published criteria for adding five variants (p.R2336H, p.R2355W, p.E3014K, p.G3990V and p.V4849I) to the genetic diagnostic panel for MH susceptibility and demonstrates equipoise among methods used to transfect and select cells. This thereby extends the availability of DNA diagnosis by 14% of MH families. We were unable to confirm the

functional significance of the p.D3986E *RYR1* variant in MH susceptibility but this may reflect an inadequacy of the heterologous HEK293 expression system for analysing all *RYR1* variants that have affected IVCT phenotypes.

Contribution of authors

Conception and design of the study: PMH, M-AS, KMS, PDA & DSS
Conduct of experiments and data collection: AM, PB, DMM, CD, DSS, PMH
Data analysis & interpretation: all authors
Drafting of manuscript: AM, PB, PMH
All authors reviewed drafts of the manuscript and approved the final version

Declaration of Interests

PMH is an Editorial Board Member and Trustee of BJA. He is also Chair of the European Malignant Hyperthermia Group

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Table 1. Summary of segregation analyses in malignant hyperthermia families where one of six recurrent *RYR1* variants has been identified. "-" indicates that it was not possible to calculate a probability of segregation using the SISA method because there were affected individuals who were not carriers of the familial variant.

	RYR1 variant					
	c.7007G>A, p.R2336H	c.7063C>T, p.R2355W	c.9310G>A, p.E3104K	c.11958C>G, p.D3986E	c.11969G>T, p.G3990V	c.14545G>A, p.V4849I
No. of families segregating	6	6	4	5	10	5
Total no. of informative meioses in segregating families	9	10	5	4	10	26
No. of non-segregating families	2	1	0	0	1	3
Probability of segregation by chance	-	-	0.031	0.0625	-	-

Figure Legends

Figure 1.

a. Representative western blot image of HEK293 cells stably transfected with wild type and mutant pc*RYR1 c*onstructs. Proteins were run on a 4-8% polyacrylamide gel and blotted overnight onto a PVDF membrane. The membrane was probed for RyR1 using a monoclonal antibody.

1b. Representative traces of HEK293 cells stably transfected with wild type and mutant pc*RYR1* constructs. The response at each caffeine concentration is seen as a temporary increase in fluorescence. The red arrows mark the time that each caffeine concentration was added to the well and instantly perfused off. Wild type transfected cells did not reach a maximum response before the addition of 5 mM caffeine. This was the same for cells transfected with the p.D3986E variant, however, cells transfected with both the p.R2336H and p.G3990V variants often reached a maximum response at caffeine concentrations as low as 2 mM.

1c-f. Caffeine concentration-response curves for HEK293 cells stably transfected with wild type and mutant pc*RYR1* constructs. (A-C) Concentration response curve for each variant stably transfected into HEK293 cells plotted with wild type responses. Data are mean (SE), n= 6 experiments in each case). (D) Box plots of the EC_{50} values calculated for each experiment. The boxes represent the inter-quartile range with the median value represented as the horizontal line within the box: the whiskers define the range. A two-tailed student's t-test showed a statistically significant reduction in EC_{50} as compared to wild type for p.R2336H and p.G3990V. No statistical significance was seen between wild type and p.D3986E. ***P<0.001

Figure 2

a. Representative western blot image of HEK293 cells transiently transfected with wild type and mutant pc*RYR1* cDNA constructs. Proteins were run on a 4-8% polyacrylamide gel and

blotted overnight onto a PVDF membrane. The membrane was probed for RyR1 using a monoclonal antibody.

b. Representative traces of caffeine-induced calcium release of cells transiently transfected with wild type and mutant pc*RYR1* cDNA constructs. All three of the mutant pc*RYR1* constructs produced a maximum response at lower caffeine concentrations than the equivalent wild type experiment. All cells responded at 1mM caffeine but the response was greater in the cells expressing mutant RyR1.

c-f Caffeine concentration-response for HEK293 cells transiently transfected with wild type and mutant pc*RYR1* constructs. (A-C) Concentration response curve for each variant transiently transfected into HEK293 cells plotted with wild type responses. Data are mean (SE), n= 6 or 7 experiments in each case). (D) Box plots of the EC_{50} values calculated for each experiment. The boxes represent the inter-quartile range with the median value represented as the horizontal line within the box: the whiskers define the range. A two-tailed student's t-test showed a statistically significant reduction in EC_{50} as compared to wild type for each mutant. *P<0.05

Figure 3

a. Representative western blot image of HEK293 cells transiently transfected with pTUNE*RYR1* and induced with 25 μM IPTG. The first lane is from transfected cells not induced with IPTG. Proteins were run on a 4-8% polyacrylamide gel and blotted overnight onto a PVDF membrane. The membrane was probed for RyR1 using a monoclonal antibody.

b. Representative traces of HEK293 cells transfected with pTUNE*RYR1* constructs showing the release of calcium when exposed to incremental doses of caffeine. Wild type transfected cells responded minimally to 1mM caffeine and had an increasing response as the concentration of caffeine increased. A maximum response appeared to be reached at around 8 mM caffeine. Cells transfected with p.D3986E responded in a similar way to wild type. Cells transfected with p.R2355W reached a maximum response at around 4 mM

caffeine and tended to have a more exaggerated response at doses of caffeine lower than this.

c-e. Caffeine concentration-response curves of cells transfected with p.R2355W or p.D3986E pTUNE*RYR1* constructs compared to wild type. (A-B) Concentration-response curve for each variant plotted with wild type responses. Data are mean (SE), n= 4 experiments in each case). (C) Box plots of the EC_{50} values calculated for each experiment. The boxes represent the inter-quartile range with the median value represented as the horizontal line within the box: the whiskers define the range. A two-tailed student's t-test showed a statistically significant reduction in EC_{50} for p.R2355W as compared to wild type. *P<0.05

Supplementary figure 1.

Pedigrees of the seven malignant hyperthermia (MH) families where affected non-carriers of familial *RYR1* variants were found. Squares represent males and circles females: a diagonal line through the symbol indicates the person is deceased. Black filled symbols with a letter "S" indicate an MH susceptible phenotype determined by *in vitro* contracture testing (IVCT). Green filled symbols with a letter "N" indicate an MH negative phenotype determined by IVCT. Unfilled symbols with a "?" indicate an individual who has not undergone IVCT. Where an individual has undergone genotyping for the familial RYR1 variant, the genotype is presented in capital letters under the individual: blue lettering indicates concordance between genotype and phenotype; red lettering indicates discordance. The red arrows identify the index case who had the clinical reaction.

Figure 1

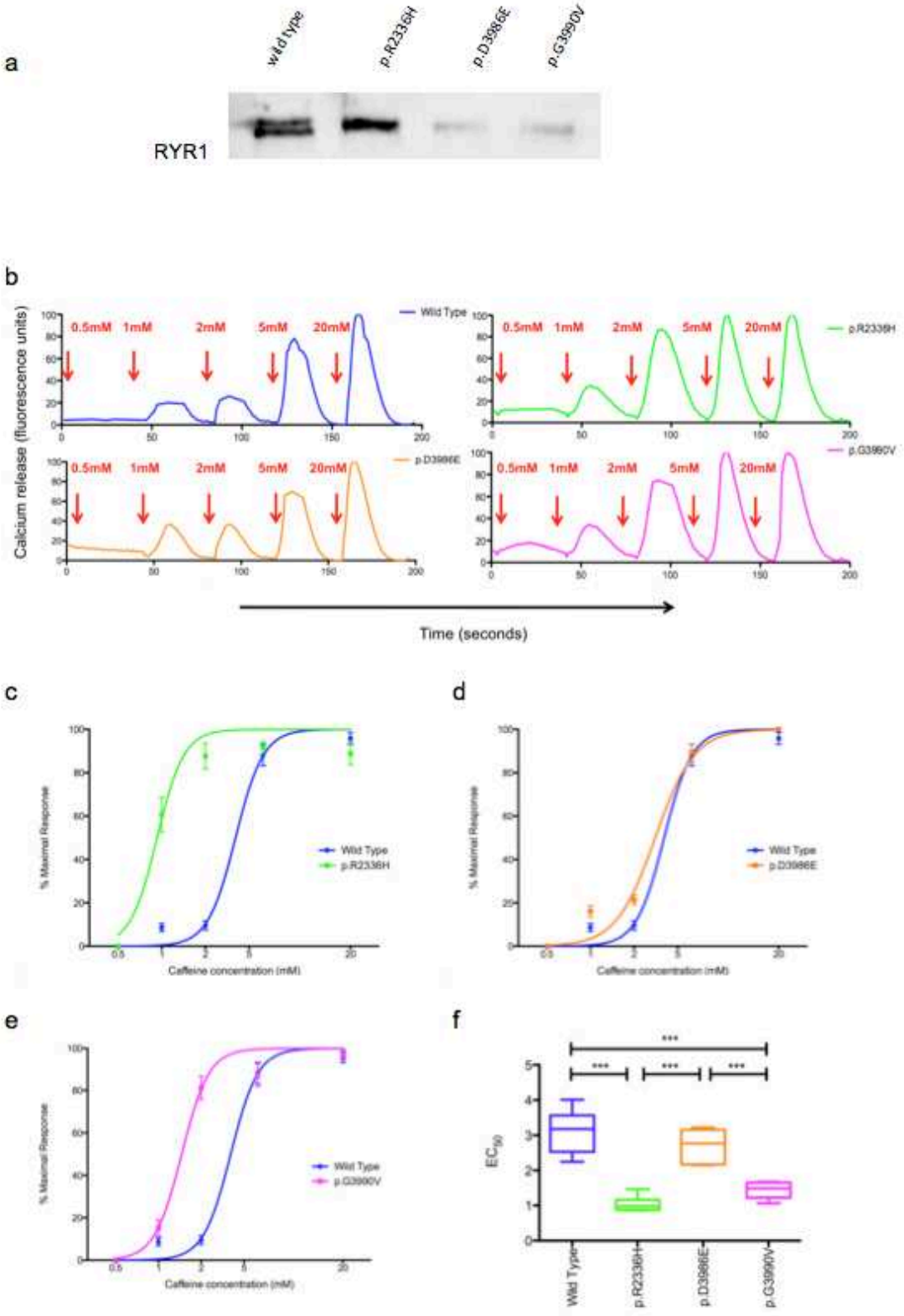

a
wild type
p.R2336H
p.D3986E
p.G3990V
RYR1
b
Calcium release (fluorescence units)
0.5mM
1mM
2mM
5mM
20mM
Wild Type
p.R2336H
p.D3986E
p.G3990V
Time (seconds)
c
% Maximal Response
Caffeine concentration (mM)
Wild Type
p.R2336H
d
% Maximal Response
Caffeine concentration (mM)
Wild Type
p.D3986E
e
% Maximal Response
Caffeine concentration (mM)
Wild Type
p.G3990V
f
EC50
***
Wild Type
p.R2336H
p.D3986E
p.G3990V

Figure 2

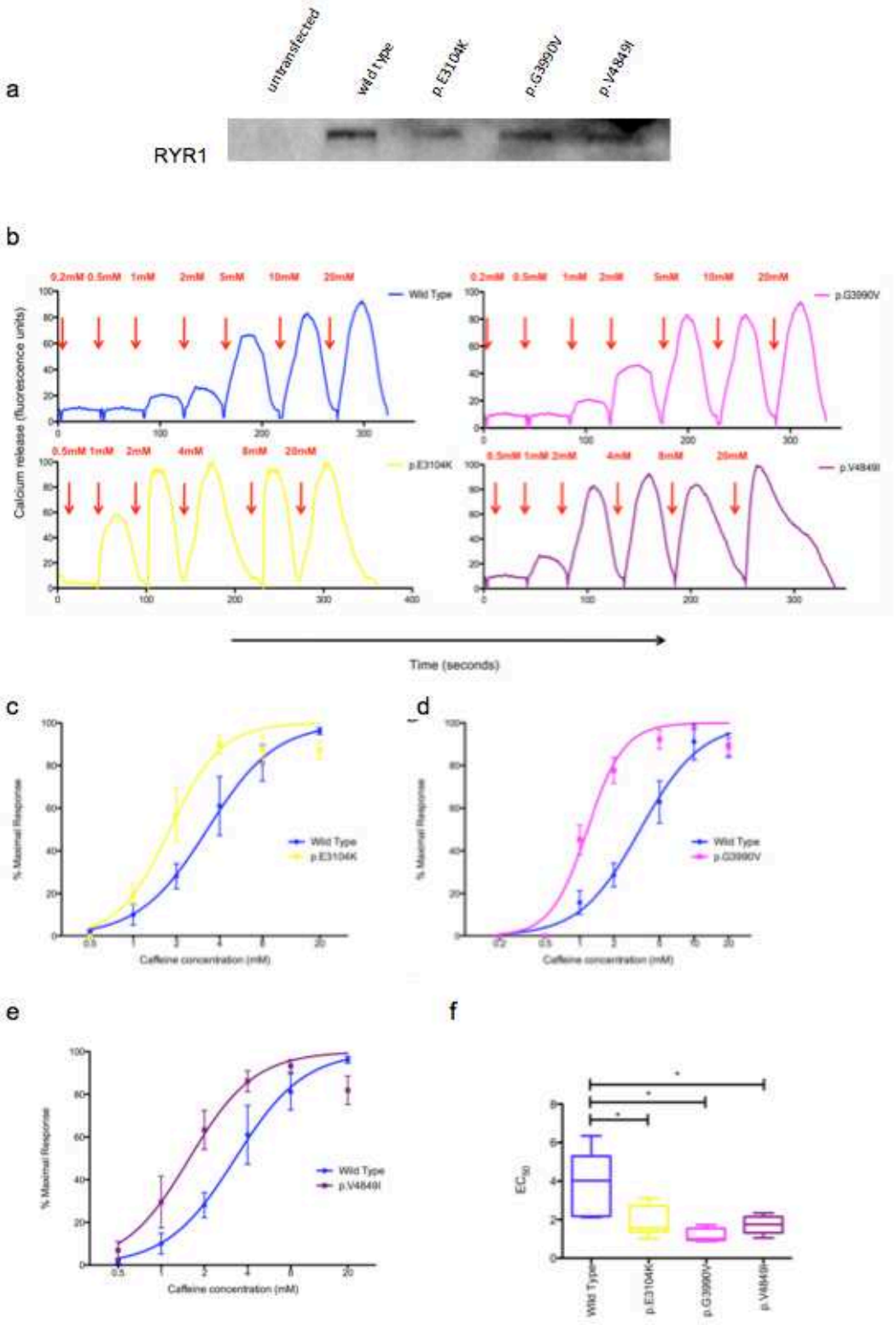
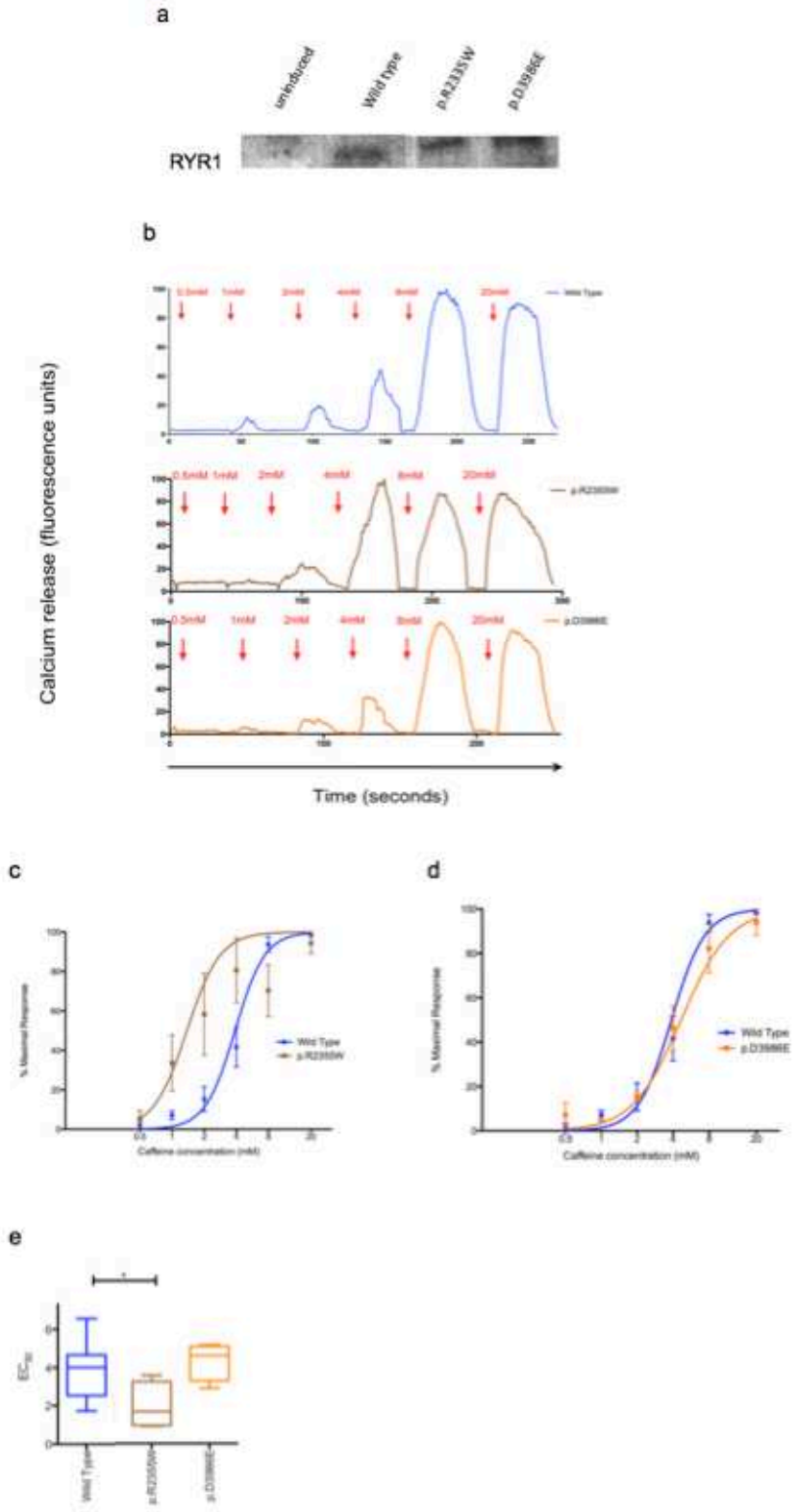

a
untransfected
wild type
p.E3104K
p.G3990V
p.V4849I
RYR1
b
Calcium release (fluorescence units)
Wild Type
p.G3990V
p.E3104K
p.V4849I
Time (seconds)
c
% Maximal Response
Caffeine concentration (mM)
Wild Type
p.E3104K
d
% Maximal Response
Caffeine concentration (mM)
Wild Type
p.G3990V
e
% Maximal Response
Caffeine concentration (mM)
Wild Type
p.V4849I
f
EC50
Wild Type
p.E3104K
p.G3990V
p.V4849I

Figure 3


a
uninduced
Wild type
p.R2355W
p.D3986E
RYR1
b
Calcium release (fluorescence units)
Time (seconds)
c
% Maximal Response
Wild Type
p.R2355W
Caffeine concentration (mM)
d
% Maximal Response
Wild Type
p.D3986E
Caffeine concentration (mM)
e

Supplementary table 1. Evolutionary conservation of the nucleotide base and amino acid affected in the variants across species. All amino acids are maintained throughout various species. The only discrepancy is the presence of a T residue instead of a C for the p.D3986E variant however the amino acid remains aspartic acid in the wild type sequence.

		cDNA change (Protein change)					
		c.7007 G (p.R2336)	c.7063 C (p.R2355)	c.9310 G (p.E3104)	c.11958 C (p.D3986)	c.11969 G (p.G3990)	c.14545 G (p.V4849)
Species	Human	G (R)	C (R)	G (E)	C (D)	G (G)	G (V)
	Pig	G (R)	C (R)	G (E)	C (D)	G (G)	G (V)
	Rabbit	G (R)	C (R)	G (E)	C (D)	G (G)	G (V)
	Mouse	G (R)	C (R)	G (E)	T (D)	G (G)	G (V)
	Chimp	G (R)	C (R)	G (E)	C (D)	G (G)	G (V)
	Dog	G (R)	C (R)	G (E)	C (D)	G (G)	G (V)

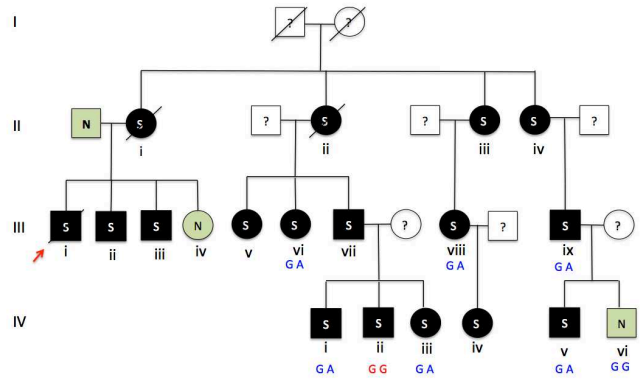
Supplementary table 2. Evolutionary conservation of the nucleotide and amino acid affected in the variants across the human *RYR* isoforms. All amino acids are maintained throughout the three RyR isoforms in humans apart from the p.R2355 residue in RyR3 which has a lysine instead of arginine. The overall importance of this particular change is unknown due to the slightly different roles these isoforms play in the different tissues they are expressed in. Similarly to the mouse *RYR1* sequence, the human *RYR2* sequence has a T residue instead of the C residue seen at position c.11958 for the p.D3986E variant, however once again the aspartic acid residue is maintained in the wild type sequence.

		cDNA change (Protein change)					
		c.7007 G (p.R2336)	c.7063 C (p.R2355)	c.9310 G (p.E3104)	c.11958 C (p.D3986)	c.11969 G (p.G3990)	c.14545 G (p.V4849)
Isoforms	RYR1	G (R)	C (R)	G (E)	C (D)	G (G)	G (V)
	RYR2	G (R)	A (R)	G (E)	T (D)	G (G)	G (V)
	RYR3	G (R)	A (K)	G (E)	C (D)	G (G)	G (V)

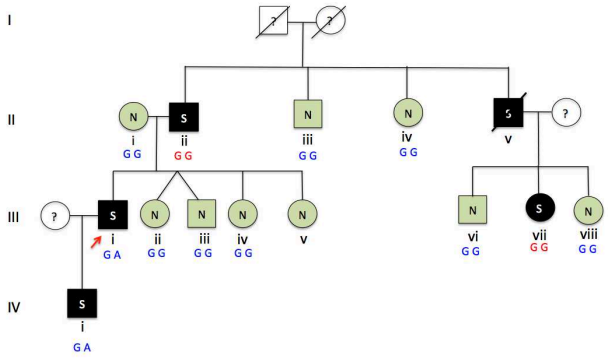
Supplementary table 3. Amino acid properties and minor allele frequencies (MAFs) of the variants selected for study. Four of the missense variants under investigation result in a substitution of amino acid that has altered properties and may therefore result in a change in the structure and function of the overall protein. Of the 6 selected variants, 2 possess no obvious changes (p.D3986E and p.V4849I). The MAFs are those reported for all populations included in the Exome Variant Server database (http://evs.g.s.washington.edu/EVS, last accessed 19.10.2016): a "-" indicates that the variant is not reported in the EVS.

Residue	Starting amino acid	Substituted amino acid	Charge	Acid/basic	Hydrophobic/ hydrophilic	MAF %
2336	Arginine	Histidine	No change (positive)	No change (basic)	Hydrophilic to neutral	-
2355	Arginine	Tryptophan	Positive to neutral	Basic to neutral	Hydrophilic to hydrophobic	0.0154
3104	Glutamic acid	Lysine	Negative to positive	Acidic to basic	No change (hydrophilic)	-
3986	Aspartic acid	Glutamic acid	No change (negative)	No change (acidic)	No change (hydrophilic)	-
3990	Glycine	Valine	No change (neutral)	No change (neutral)	Neutral to hydrophobic	-
4849	Valine	Isoleucine	No change (neutral)	No change (neutral)	No change (hydrophobic)	-

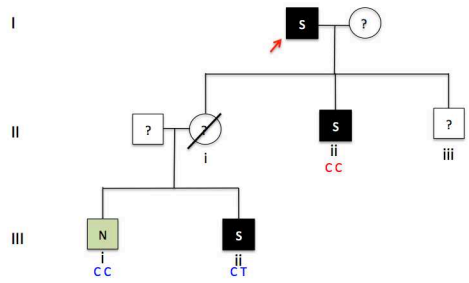
Supplementary figure



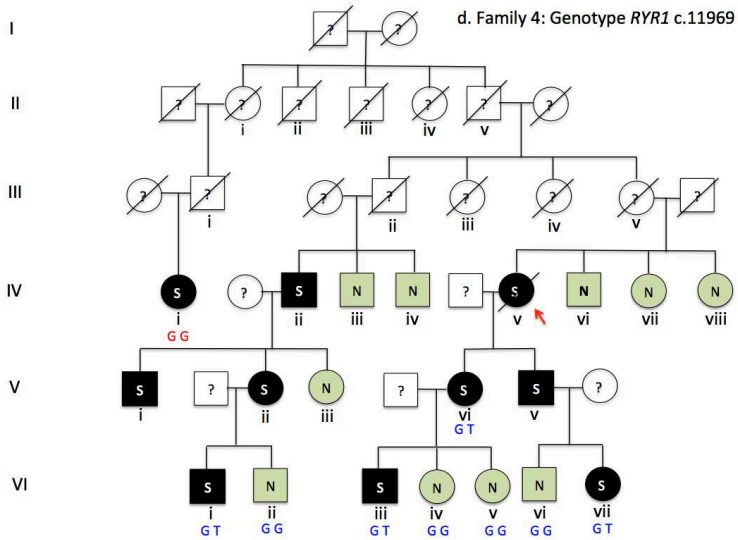
a. Family 1: Genotype *RYR1* c.7007



b. Family 2: Genotype *RYR1* c.7007

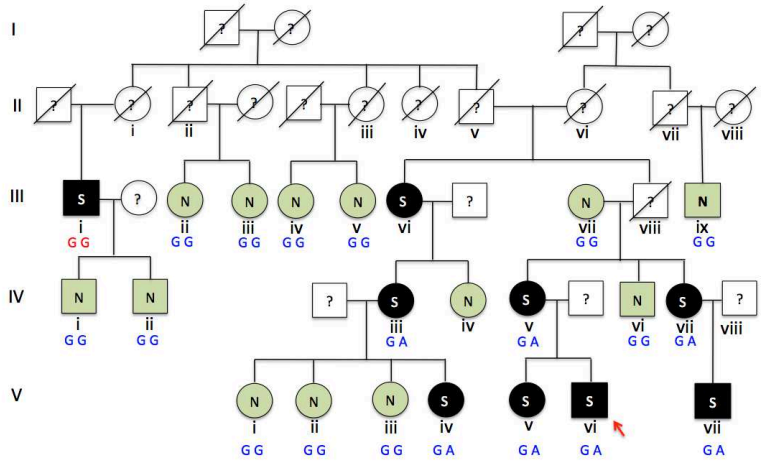


c. Family 3: Genotype *RYR1* c.7063

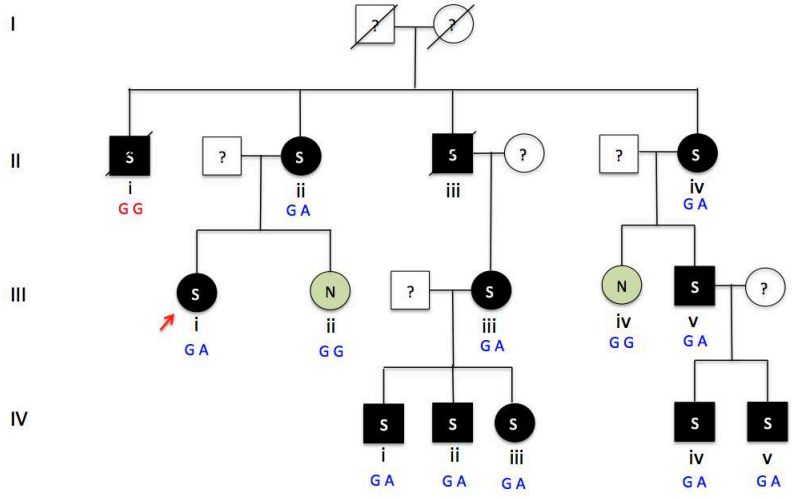


d. Family 4: Genotype *RYR1* c.11969

e. Family 5: Genotype *RYR1* c.14545



f. Family 6: Genotype *RYR1* c.14545



g. Family 7: Genotype *RYR1* c.14545