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Genetic variation in *RYR1* and malignant hyperthermia phenotypes

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Background. Malignant hyperthermia (MH) is associated, in the majority of cases, with mutations in *RYR1*, the gene encoding the skeletal muscle ryanodine receptor. Our primary aim was to assess whether different *RYR1* variants are associated with quantitative differences in MH phenotype.

Methods. The degree of *in vitro* pharmacological muscle contracture response and the baseline serum creatine kinase (CK) concentration were used to generate a series of quantitative phenotypes for MH. We then undertook the most extensive *RYR1* genotype–phenotype correlation in MH to date using 504 individuals from 204 MH families and 23 *RYR1* variants. We also determined the association between a clinical phenotype and both the laboratory phenotype and *RYR1* genotype.

Results. We report a novel correlation between the degree of *in vitro* pharmacological muscle contracture responses and the onset time of the clinical MH response in index cases ($P<0.05$). There was also a significant correlation between baseline CK concentration and clinical onset time ($P=0.039$). The specific *RYR1* variant was a significant determinant of the severity of each laboratory phenotype ($P<0.0001$).

Conclusions. The MH phenotype differs significantly with different *RYR1* variants. Variants leading to more severe MH phenotype are distributed throughout the gene and tend to lie at relatively conserved sites in the protein. Differences in phenotype severity between *RYR1* variants may explain the variability in clinical penetrance of MH during anaesthesia and why some variants have been associated with exercise-induced rhabdomyolysis and heat stroke. They may also inform a mutation screening strategy in cases of idiopathic hyperCKaemia.

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Malignant hyperthermia (MH) is a potentially life-threatening pharmacogenetic disorder. During an MH crisis, potent inhalation anaesthetics and the neuromuscular blocking agent succinylcholine trigger a massive accumulation of calcium within skeletal muscle cells, which in turn leads to acceleration of muscle metabolism and contractile activity. This generates heat and leads to hypoxaemia, metabolic acidosis, rhabdomyolysis, and a rapid increase in body temperature, which can be fatal if not recognized and treated promptly.

Increased sensitivity of muscle biopsy specimens to the contracture-inducing properties of halothane and caffeine was discovered more than 30 yr ago.[1 2] These findings both formed the basis of diagnostic tests for MH (*in vitro* contracture tests, IVCT) and indicated the defect to lie in the excitation–contraction coupling mechanism.[3] Defects in the ryanodine receptor protein (RyR1), which forms the Ca^{2+} release channel of the sarcoplasmic reticulum of

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skeletal muscle, have been implicated in more than 50% of MH cases in the UK.[4] There have been 178 non-synonymous changes with potential functional significance described across the *RYR1* gene.[5] Of these, 72 have been documented to be recurrent,[5] and 29 have been functionally characterized in *in vitro* studies and can thus be referred to as mutations (www.emhg.org and Yang and colleagues).[6] We refer to changes in the *RYR1* sequence that are potential mutations but have not been functionally characterized as variants. Ten of the 29 functionally characterized mutations have also been described in patients suffering from central core disease (CCD), a congenital myopathy presenting with muscle weakness, and with characteristic cores lacking oxidative enzymes on histochemical analysis. Patients with CCD are often susceptible to MH, although the segregation of MH and CCD is not always concordant within families.[7]

The considerable variability in the clinical presentation of MH during anaesthesia has been implicated in delayed diagnosis, resulting in severe morbidity and mortality.[8] Our hypothesis, based on a preliminary assessment,[9] is that different *RYR1* variants confer differential sensitivity to the triggering agents, thereby contributing to the variability in clinical presentation. Similarly, it may be those *RYR1* mutations with more profound functional consequences that are associated with the co-occurrence of MH with CCD[10] or 'idiopathic' hyperCKaemia.[11]

Our primary aim, therefore, was to assess whether different *RYR1* variants are associated with quantitative differences in MH phenotype. To produce quantitative phenotypes, we used data from the diagnostic IVCT and serum creatine kinase (CK) concentration. The clinical relevance of the quantitative IVCT phenotype was assessed by analysing its association with the speed of onset of the clinical reaction in index cases. In further analyses, we assessed the following for their influence on severity of phenotype: whether or not the variant has been described with the CCD phenotype in addition to MH; the extent of evolutionary conservation at each specific variant location; and the degree of conservation at each variant site across the RyR isoforms. The present study utilizes the largest resource worldwide of phenotypically and genotypically characterized MH patients, to generate the most extensive *RYR1* genotype–IVCT phenotype correlation in MH to date using 504 individuals from 204 MH families and 23 *RYR1* variants.

Methods

Patients and diagnosis of MH susceptibility

The patients included in this study attended the UK MH Investigation Unit at St James's University Hospital, Leeds, UK, for determination of their susceptibility to MH. The patients either had had a suspected clinical MH reaction (proband or index cases) themselves, were the closest relative to a proband who was unable to be tested (deceased, medically unfit, or too young), or were related to individuals previously diagnosed susceptible to MH. The study was approved by the Leeds East Local Research Ethics Committee, and patients gave written informed consent.

Laboratory diagnosis of MH is through the IVCT, for which the European and North American MH groups have well-defined and standardized protocols. The IVCT method has been used for 35 yr and is used in Europe for the initial assessment for predisposition to MH susceptibility of index cases because of its high sensitivity.[12] All individuals in this study were diagnosed by the IVCT according to the European MH group guidelines (www.emhg.org). This involves exposure of skeletal muscle biopsy specimens (from vastus medialis) to incremental concentrations of halothane or caffeine in an irrigated tissue bath, and measurement of muscle contracture in response to the applied stimulants. The European protocol assigns the patient to one of the three laboratory diagnostic categories, MHS, MHN, or MHE, according to whether their muscle displays increased sensitivity to both, none, or only one of the stimulants, respectively. Both MHS and MHE categories are considered to represent clinical susceptibility to MH. Patients included in this study were MHS or MHE. Muscle specimens taken at the time of biopsy were examined by histopathologists to assess for the presence or absence of CCD. Individuals who presented clinically and histologically with both CCD and MH phenotypes were excluded from this study in order to focus the analysis solely on the MH phenotype.

Generation of quantitative phenotype data

In addition to the qualitative difference in phenotype implicit in separate MHS and MHE categories, there is a wide range in magnitude of contracture responses between individuals in each of these categories. It is not clear if such differences are associated with variability in clinical MH responses, different causative genetic factors, or both. To generate quantitative phenotypes, we used data derived from the three diagnostic components of the IVCT used in our laboratory: static halothane, static caffeine, and dynamic halothane tests. In the 'static' tests, halothane or caffeine is added after an equilibration period during which the specimen reaches a stable tension after being stretched to its optimum physiological length. In the dynamic halothane test, each concentration of halothane is added during a timed stretch/relaxation manoeuvre and responses compared with stretch/relaxation manoeuvres done before application of the drug. The phenotype for the halothane test responses was defined as the contracture response (g) at halothane 2% (v/v), whereas the phenotype

for the static caffeine test was the contracture response (g) at caffeine 2 mM.

A fourth quantitative IVCT phenotype was derived from a ryanodine contracture test,[13] which we do when sufficient viable tissue is available. In this case, the phenotype was defined as the time (min) following addition of ryanodine 1 μM to the onset of contracture. The serum CK concentration (IU litre^{-1}) was used as a further quantitative phenotype, the blood sample being drawn immediately before the muscle biopsy. Before the muscle biopsy, all patients are starved overnight with avoidance of physical exertion and heavy alcohol consumption for at least 72 h before testing. Probands are not tested for at least 4 months after their clinical reaction to allow resolution of any muscle damage.

Onset of clinical reaction

In order to explore the relevance of the quantitative phenotypes, the rapidity of the development of the clinical reaction was documented for probands. The time to clinical onset of the MH reaction was defined as the time, in minutes, from the start of anaesthesia to the development of two of the following: evidence of inappropriate increased carbon dioxide production, inappropriate increase in heart rate, or inappropriate increase in core body temperature. The clinical onset data were recorded directly from the contemporaneous anaesthesia records by a single investigator (P.M.H.) blinded to the IVCT contracture strengths and the nature of the *RYR1* variant. Patients whose only feature was muscle rigidity after succinylcholine were excluded from this analysis, as this sign is difficult to quantify clinically and represents succinylcholine sensitivity (an indicator of risk of MH susceptibility) rather than an MH reaction *per se*.

RYR1 screening and variant analysis

DNA from 450 MH-susceptible individuals from independent families was screened using assays previously described (www.emhg.org) for 12 functionally characterized mutations that were known to be recurrent in the UK MH population (p.R163C, p.G248R, p.G341R, p.R614C, p.R2163H, p.V2168M, p.T2206M, p.A2350T, p.G2434R, p.R2435H, p.R2454H, and p.R2458H). *RYR1* cDNA sequencing was performed on 95 independent MHS individuals, who did not have these or 16 other functionally characterized *RYR1* mutations [www.emhg.org]. cDNA prepared from total RNA isolated from muscle biopsy specimens was used to sequence the ~15 kb *RYR1* cDNA, using 25 overlapping fragments of ~700 bp in length, read in both the forward and the reverse directions, and analysed on an ABI3730. Eleven non-synonymous recurrent changes identified in this patient panel were subsequently used to screen unrelated UK MH patients using assays developed in-house (detailed in Table 1). Where variants were detected, extended pedigrees were investigated to assess the concordance between MH susceptibility (as defined by the diagnostic IVCT) and *RYR1* genotype. Where discordance was detected, this was confirmed by testing an independent DNA aliquot. None of the variants was detected on screening 200 independent normal controls.

Data handling and statistical analyses

Quantitative phenotype data

The distributions of the quantitative IVCT phenotype and CK concentration data were all significantly skewed and these variables were transformed by a Box–Cox transformation before further analysis. The residuals after each

Table 1 Details of the detection assays designed in-house for the 11 recurrent *RYR1* variants identified through sequencing

Exon	Mutation		Primers		
6	c.529C>T	p.R177C	F: TTC TGG GAA GCC ATC ATC TG R: GAC CTG GGA GCA GGA GGA AC	*Hha*I loss	WT: 197 bp cleaves to 157 and 40 bp
34	c.5183 C>T	p.S1728F	F: ATG TGA TGA AGG AAA TGG AG R: TTG ATG CAT GTA TCT CTG GA	*Bsr*BI loss	WT: 781 bp cleaves to 338, 270, and 173 bp Het mutant: 781 bp cleaves to 511, 338, 270, and 173 bp
43	c.7007G>A	p.R2336H	F: GCA TGG GTC TGG TCT CTG AC R: TCA TCT CGA GGA GGT GTG TG	*Hha*I loss	WT: 247 bp cleaves to 139, 90, and 18 bp Het mutant: 247 bp cleaves to 139, 108, 90, and 18 bp
44	c.7063C>T	p.A2355W	F: ATG GGA GGT CTC TGA TGG TG R: AGA TGC GGA TGG CCT CTT	*Aci*I loss	WT: 186 bp cleaves to 53, 45, 46, and 42 bp Het mutant: 186 bp cleaves to 88, 53, 45, 46, and 42 bp
45	c.7291G>T	p.D2431Y	F: CCC CTC CTC AAT AGG CAA C R: CTG CAT GAG GCG TTC AAA G	*Taq*I loss	WT: 248 bp cleaves to 146 bp & 101 bp. Het mutant: 248 bp cleaves to 248, 146, and 101 bp
63	c.9310G>A	p.E3104K	F: CGA TTC CAG AGC TGA TGT TC R: CGC GTC CTC ATT AAC TCA TT	*Taq*I loss	WT: 442 bp cleaves to 329, 92, and 21 bp Het mutant: 442 bp cleaves to 350, 329, 92, and 21 bp
87	c.11958C>G	p.D3986E	F: GTG ATC CCT GAT CCC TTC TC R: GAA GCA GGT GGA TGG AGA C	*Eco*NI gain	WT: 200 bp Het mutant: 200 bp cleaves to 200, 108, and 92 bp
87	c.11969G>T	p.G3990V	F: GTG ATC CCT GAT CCC TTC TC R: GAA GCA GGT GGA TGG AGA C	*Hin*FI loss	WT: 200 bp cleaves to 99, 77, and 24 bp Het mutant: 200 bp cleaves to 176, 99, 77, and 24 bp
98	c.14210G>A	p.R4737Q	F: CCT GCT GCT CTC TGG TGC AG R: CCC TGC TCA CGT TGT CTG T	*Bsr*BI loss	WT: 317 bp cleaves to 261 and 56 bp Het mutant: 317 bp cleaves to 317, 261, and 56 bp
100	c.14477C>T	p.T4826I	F: TCA CAT GTA CGT GGG TTT CT R: GCA CTG ACC CTG GAT GAT G	ARMS assay	Control primers; F: TGT CAC AGT GGT GGC TAT GG R: TTG TCA CCA TCT TGG GGA AG
101	c.14545G>A	p.V4849I	F: GGA AGA GCC ACA GGG ACT GA R: GAG AAG GAA GGG TCC CAG AG	*Acc*I loss	WT: 310 bp cleaves to 260 and 50 bp Het mutant: 310 bp cleaves to 310, 260, and 50 bp

transformation were not significantly skewed, although the residuals for the static caffeine tension and static halothane tension had significant kurtosis.

Analysis of time to clinical onset

The relationships between the quantitative phenotype variables and the time to the onset of the clinical reaction were analysed using the Cox proportional hazards models, with time to onset as the dependent variable. Each IVCT variable was analysed separately, with covariates age, sex, the volatile anaesthetic agent used, and whether the patient received succinylcholine. The significance of variables was assessed using the likelihood ratio test. The relationship between the variant carried by the proband and the time to clinical onset was also analysed.

Analysis of IVCT phenotypes and genotype

The relationship between *RYR1* variant and each quantitative phenotype was examined in mixed model analyses. For each model, the quantitative phenotype was the dependent variable, and the explanatory variables were: variant (categorical, variable with 23 levels: p.R163C, p.R177C, p.G248R, p.G341R, p.R614C, p.S1728F, p.R2163H, p.V2168M, p.T2206M, p.R2336H, p.A2350T, p.R2355W, p.D2431Y, p.G2434R, p.R2435H, p.R2454H, p.R2458H, p.E3104K, p.D3986E, p.G3990V, p.R4737Q, p.T4826I, and p.V4849I); sex [categorical, 2 levels: male (n=255), female (n=249)]; and age (continuous). In addition, for each of the models generated with a quantitative IVCT phenotype as the dependent variable, three additional explanatory variables were included to assess the influence of muscle specimen characteristics. These were the length of the muscle specimen, weight of the specimen, and the pre-drug electrically evoked twitch response (all continuous variables). When a model demonstrated the variant to be a significant explanatory variable, the differences in phenotypes between p.G2434R and each of the other variants were tested by linear contrasts. The p.G2434R mutation was selected to contrast with other variants because it is the most prevalent mutation in the UK population. As more than one variant-positive individual per family was included, individuals are not independent since they share family background. To control for this non-independence, 'family' was included as a random effect nested within variant in all analyses.

Additional analysis considered the variation in phenotype according to variant type. Three new variables were created, each describing one aspect of *RYR1* variants: (i) variants previously described in association with both the MH and the CCD phenotypes (coded 1) *vs* variants described with MH only (coded 0); (ii) variants at residues conserved in RyR1 sequences from multiple species (coded 1) *vs* those conserved solely within the mammalian species considered (coded 0) [taken from all available RyR1 protein sequences from NCBI; *Homo sapiens* (P21817), *Sus scrofa* (P16960), *Mus musculus* (Q80X16), *Oryctolagus cuniculus* (P11716), *Drosophila melanogaster* (Q24498), *Caenorhabditis elegans* (P91905), *Anopheles gambiae* (Q7PJQ9), *Rana catesbeiana* (Q91313), *Milax nigricans* (O13054), and *Hemicentrotus pulcherrimus* (Q8TA74); and (iii) variants at residues conserved within all three *RYR* isoforms (coded 1) *vs* those not conserved (coded 0) (*RYR2*, chromosome 1 OMIM#180902; *RYR3*, chromosome 15, OMIM#180903). Each of these variables was examined in a separate mixed model with all covariates included, and with both variant and family included as random effects, with family nested within variant type.

All analyses were conducted using Stata 9.1 (StataCorp, College Station, TX, USA). *P*-values are presented after correction for multiple comparisons where appropriate and $P<0.05$ was considered statistically significant.

Results

Analysis of IVCT and clinical onset

Sufficient data were available for 62 probands. The median time to clinical onset in these individuals was 30 min (range 5–240 min). There was a significant association between a shorter time to onset of the clinical reaction and the severity of each quantitative IVCT phenotype except the dynamic halothane tension. There was also a significant association between CK concentration and time to onset (Table 2). There was also an association between longer clinical onset times and increasing age of the probands ($P<0.001$). However, there was no significant relationship between the particular volatile anaesthetic received or the use of succinylcholine and time to clinical onset. The median time to clinical onset varied from 10 to 135 min for different variants (Table 3), but there was no significant relationship between variant and time to clinical onset ($P=0.75$).

RYR1 screening and variant analysis

Screening for functionally characterized mutations

Individuals from 450 independent MH families were screened for 12 functionally characterized *RYR1* mutations recurrent in the UK population. The number of families found to carry each of these mutations was: p.R163C (n=13), p.G248R (n=3), p.G341R (n=17), p.R614C (n=6),

Table 2 Relationship between time to clinical onset (min) for probands and MH IVCT phenotypes. Hazard ratios from Cox proportional hazards regression were adjusted for age, sex, and succinylcholine usage

	n	Adjusted HR (95% CL)	*P*-value
Static caffeine tension	61	1.640 (1.068–2.517)	0.027
Static halothane tension	62	1.228 (1.018–1.482)	0.043
Dynamic halothane tension	62	1.236 (0.965–1.584)	0.10
Static ryanodine tension	53	0.933 (0.886–0.983)	0.0057
CK	57	1.001 (1.000–1.002)	0.039

Table 3 Variation in time to clinical onset for probands according to *RYR1* variant. *The number of probands with each variant and full clinical data. †Minimum observed time to clinical reaction for the particular variant. ‡Maximum observed time to clinical reaction for the particular variant. §Median time to clinical reaction for the particular variant

Codon change	Amino acid change	Probands*	Minimum† (min)	Maximum‡ (min)	Median§ (min)
c.487C>T	p.R163C	6	5	45	27.5
c.529C>T	p.R177C	5	15	240	50
c.742G>A	p.G248R	0			
c.1021G>A	p.G341R	6	10	60	15
c.1840C>T	p.R614C	3	10	100	60
c.5183C>T	p.S1728F	0			
c.6488G>A	p.R2163H	0			
c.6502G>A	p.V2168M	2	20	60	40
c.6617C>T	p.T2206M	5	10	90	30
c.7007G>A	p.R2336H	2	60	60	60
c.7048G>A	p.A2350T	2	10	10	10
c.7062C>T	p.R2355W	0			
c.7291G>T	p.D2431Y	0			
c.7300G>A	p.G2434R	32	10	120	27.5
c.7304G>A	p.R2435H	2	30	45	37.5
c.7361G>A	p.R2454H	1	40	40	40
c.7373G>A	p.R2458H	2	10	20	15
c.9310G>A	p.E3104K	0			
c.11958C>G	p.D3986E	2	25	210	117
c.11969G>T	p.G3990V	1	45	45	45
c.14210G>A	p.R4737Q	1	10	10	10
c.14477C>T	p.T4826I	1	135	135	135
c.14545G>A	p.V4849I	1	25	25	25
Total		74			

p.R2163H (n=8), p.V2168M (n=6), p.T2206M (n=22), p.A2350T (n=3), p.G2434R (n=90), p.R2435H (n=8), p.R2454H (n=9), and p.R2458H (n=10).

RYR1 cDNA sequencing

Screening of 95 independent MHS samples identified non-synonymous changes in *RYR1* in a total of 67 individuals, 70% of cases. These included 11 recurrent non-synonymous changes that were new to the UK; five have been previously reported (p.R177C,[14] p.R2355W,[15] p.R4737Q,[14] p.T4826I,[16] and p.V4849I);[17] we reported the other six for the first time in a recent review (p.S1728F, p.R2336H, p.D2431Y, p.E3104K, p.D3986E, and p.G3990V).[5] A further 450 independent UK MHS samples were screened for these 11 recurrent mis-sense changes, giving the total number of UK MH families with each of these 11 variants as p.R177C (n=7), p.S1728F (n=7), p.R2336H (n=7), p.R2355W (n=6), p.D2431Y (n=2), p.E3104K (n=4), p.D3986E (n=3), p.G3990V (n=8), p.R4737Q (n=6), p.T4826I (n=10), and p.V4849I (n=5).

RYR1 genotype–IVCT phenotype correlation

Patients were included in this analysis, if they had one of the 23 recurrent variants described above and complete data from specimens meeting the EMHG IVCT protocol viability criteria (www.emhg.org) for each of the static caffeine, static halothane, and dynamic halothane tests. There were 504 individuals from 204 families who met these criteria (Table 4). There was only a single family with the variant p.D2431Y that had full IVCT data; this variant was therefore excluded from further analysis giving a total of 22 variants in the statistical analysis of 203 families and 502 individuals.

Mixed model analyses of the relationship between *RYR1* variant and each quantitative phenotype (Table 5) found that all four IVCT phenotypes, and CK concentration, differed significantly with variant. Phenotypes for the dynamic halothane test and for CK were significantly less severe with increasing age, and significantly more severe in males. The pre-drug twitch response was significantly positively correlated with all IVCT phenotypes and the length of the muscle specimen was significantly positively correlated with all except the static caffeine test. The weight of the muscle specimen was significantly positively correlated with the ryanodine test onset time.

The most prevalent variant in the UK, p.G2434R, was used as a comparator for all other variants because the phenotype resulting from p.G2434R has previously been described as relatively mild (weaker contractures/longer response time). This variant has been functionally characterized and shown to have a heightened sensitivity to activating concentrations of Ca^{2+} and to caffeine and 4-chloro-*m*-cresol *in vitro*.[18] Comparison between p.G2434R and all the other variants for the quantitative IVCT phenotypes and the CK concentration showed that the variants p.R163C, p.R2163H, p.R2435H, and p.T4826I were associated with more severe IVCT reactions (stronger contractures/shorter response times) and higher CK concentrations than p.G2434R (Fig. 1). In contrast, p.S1728F was significantly associated with weaker quantitative IVCT phenotypes than p.G2434R. p.R2454H was

Table 4 Characteristics of the non-synonymous recurrent *RYR1* variants in the UK with IVCT data. *The number of individuals with each variant and full IVCT data. †Species alignments of all available full RYR1 protein sequences from NCBI. Species used were *H. sapiens* (P21817), *S. scrofa* (P16960), *M. musculus* (Q80X16), *O. cuniculus* (P11716), *D. melanogaster* (Q24498), *C. elegans* (P91905), *A. gambiae* (Q7PJQ9), *R. catesbeiana* (Q91313), *M. nigricans* (O13054), and *H. pulcherrimus* (Q8TA74). The comment 'in all species' refers to a site conserved within all species included in this alignment, and 'in mammals' refers to conservation only within the mammals included in the analysis. ‡Isoform alignments were done between RYR1 (OMIM#180901), RYR2 (OMIM#180902), RYR3 (OMIM#180903) protein sequences. Sites were described as conserved when they were preserved in all three isoforms, otherwise the site was described as not conserved

Codon change	Amino acid change	I*	No. families	Condition	Level of conservation†	Conserved in RYR isoforms‡
c.487C>T	p.R163C	16	7	MH/CCD	In all species	Yes
c.529C>T	p.R177C	19	7	MH	In all species	Yes
c.742G>A	p.G248R	5	3	MH	In mammals	No
c.1021G>A	p.G341R	26	14	MH	In all species	Yes
c.1840C>T	p.R614C	13	6	MH/CCD	In all species	Yes
c.5183C>T	p.S1728F	6	2	MH	In mammals	No
c.6488G>A	p.R2163H	15	7	MH/CCD	In all species	Yes
c.6502G>A	p.V2168M	8	3	MH	In all species	Yes
c.6617C>T	p.T2206M	43	20	MH	In mammals	Yes
c.7007G>A	p.R2336H	13	6	MH	In all species	Yes
c.7048G>A	p.A2350T	6	3	MH	In all species	Yes
c.7062C>T	p.R2355W	14	5	MH	In all species	No
c.7291G>T	p.D2431Y	2	1	MH	In all species	Yes
c.7300G>A	p.G2434R	181	73	MH	In mammals	Yes
c.7304G>A	p.R2435H	14	6	MH/CCD	In mammals	Yes
c.7361G>A	p.R2454H	11	3	MH	In all species	Yes
c.7373G>A	p.R2458H	22	7	MH	In mammals	Yes
c.9310G>A	p.E3104K	8	4	MH	In mammals	Yes
c.11958C>G	p.D3986E	5	3	MH	In all species	Yes
c.11969G>T	p.G3990V	4	3	MH	In all species	Yes
c.14210G>A	p.R4737Q	17	6	MH	In mammals	Yes
c.14477C>T	p.T4826I	30	10	MH	In mammals	Yes
c.14545G>A	p.V4849I	26	5	MH/CCD	In mammals	Yes
Total		504	204			

Table 5 Analysis of the relationships between MH-related phenotypes and *RYR1* variant, age, sex, and muscle specimen characteristics. χ^2 and *P*-values from a mixed model analysis with family background included as a random effect. Figures in bold represent statistically significant results of $P<0.05$

Explanatory variable	Dependent variable				
	Static caffeine tension ($n=499$)	Static halothane tension ($n=499$)	Dynamic halothane tension ($n=500$)	Static ryanodine time ($n=445$)	Creatine kinase ($n=462$)
RYR1 variant	$\chi^2_{(21)}=91.70$; **$P<0.0001$**	$\chi^2_{(21)}=47.43$; **$P<0.0001$**	$\chi^2_{(21)}=63.15$; **$P<0.0001$**	$\chi^2_{(21)}=135.6$; **$P<0.0001$**	$\chi^2_{(21)}=69.64$; **$P<0.0001$**
Sex	$\chi^2_{(1)}=1.50$; $P=0.221$	$\chi^2_{(1)}=1.00$; $P=0.318$	$\chi^2_{(1)}=8.27$; **$P=0.004$**	$\chi^2_{(1)}=2.97$; $P=0.085$	$\chi^2_{(1)}=74.12$; **$P<0.0001$**
Age	$\chi^2_{(1)}=0.03$; $P=0.864$	$\chi^2_{(1)}=0.39$; $P=0.532$	$\chi^2_{(1)}=5.90$; **$P=0.015$**	$\chi^2_{(1)}=0.09$; $P=0.766$	$\chi^2_{(1)}=14.27$; **$P<0.0001$**
Specimen length	$\chi^2_{(1)}=3.14$; $P=0.076$	$\chi^2_{(1)}=15.54$; **$P<0.0001$**	$\chi^2_{(1)}=37.3$ **$P<0.0001$**	$\chi^2_{(1)}=7.73$; **$P=0.005$**	
Specimen weight	$\chi^2_{(1)}=0.13$; $P=0.722$	$\chi^2_{(1)}=0.42$; $P=0.515$	$\chi^2_{(1)}=0.06$; $P=0.811$	$\chi^2_{(1)}=4.68$; **$P=0.030$**	
Pre-test twitch	$\chi^2_{(1)}=34.14$; **$P<0.0001$**	$\chi^2_{(1)}=68.27$; **$P<0.0001$**	$\chi^2_{(1)}=10.37$; **$P=0.0013$**	$\chi^2_{(1)}=54.62$; **$P<0.0001$**	

associated with more severe static halothane ($P=0.019$), static caffeine ($P<0.0001$), and CK ($P<0.001$) phenotypes than p.G2434R. An increase in the severity of the static caffeine ($P<0.0001$) and static ryanodine ($P<0.0001$) responses with relation to p.G2434R was observed with p.G341R. p.G3990V was associated with greater static caffeine ($P=0.009$) and dynamic halothane ($P=0.019$) contractures than p.G2434R, whereas p.D3986E was associated with more severe static caffeine contractures ($P=0.002$) and greater CK concentrations ($P<0.01$) than p.G2434R. p.T2206M displayed shorter static ryanodine response times than p.G2434R ($P=0.001$). Longer ryanodine response times were observed with p.E3104K ($P=0.001$) and p.R4737Q ($P=0.001$). Indeed, there is overlap between the 95% confidence interval (CI) of static ryanodine response time for p.S1728F, p.E3104K, and p.R4737Q and that for MHN patients (24.5–27.5 min, mean 26 min).[13] Variants p.R2336H ($P<0.05$) and p.R614C ($P<0.05$) were associated with higher CK concentrations than p.G2434R. None of the variants was associated with significantly lower CK concentrations than p.G2434R. Indeed, there is no overlap of the CK concentration 95% CI for any of the variants with the 95% CI obtained from 100 MHN individuals (68–90 IU litre^{-1}, mean 79 IU litre^{-1}).

In addition to investigating each variant individually, analyses were done by grouping variants and comparing the data between the groups (Table 6). Five of the variants analysed (p.R163C, p.R614C, p.R2163H, p.R2435H, and p.V4849I) have been described as being associated with

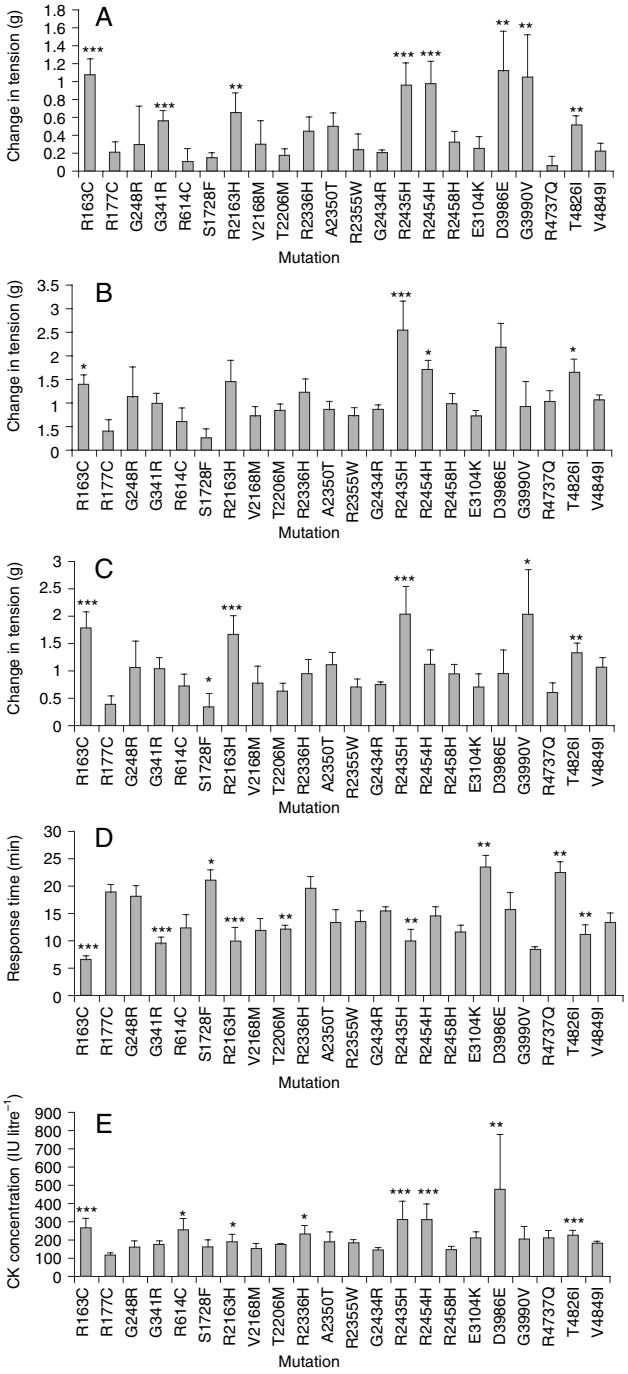

Fig 1 Graphical representation of the differences between the means for each *RYR1* variant type for every MH IVCT phenotype. (A) Static caffeine (n=502), (B) static halothane (n=502), (C) dynamic halothane (n=502), (D) static ryanodine response time (n=446), and (E) CK levels (n=463). Values shown are the mean and SE of back transformed data for each variant. Asterisks represent significant differences from G2434R; *P<0.05, **P<0.01, and ***P<0.001.

the CCD phenotype and MH (Table 4). When analysed collectively, these MH/CCD-associated variants were found to be associated with a significantly greater dynamic halothane tension (P=0.022) and a shorter static ryanodine response time (P=0.023), than those variants that have not been described in relation to CCD (Table 6).

The locations of the amino acids substituted for each variant in this analysis were investigated in relation to their evolutionary conservation within the RyR protein using all complete RyR1 sequences available on NCBI for a number of mammalian species and others (Table 4). The RyR1 protein shows 93% conservation at 4713 sites, within the four mammalian species that have their full RyR1 protein sequence available. Across all 10 species studied, ~25% of the protein was conserved. Substitution at a site conserved in all species was associated with increased severity for static caffeine tension (P=0.016) and static ryanodine response time (P=0.019), compared with those sites only showing conservation within the mammalian species investigated (Table 6).

Variants were further assessed with respect to their conservation within the protein sequence of the *RYR* isoforms, *RYR1*, *RYR2*, and *RYR3*, for which there is 56% identity between all three isoforms. The fact that there are only three sites investigated that were not conserved in all three isoforms may limit the power of this particular analysis, and no significant findings were observed with any of the IVCT-related phenotypes, nor CK concentration (Table 6).

Discussion

This study represents the largest retrospective analysis of the relationship between *RYR1* variants and MH-related phenotypes and is based on the largest resource of MH individuals with *RYR1* genotyping data and standardized IVCT phenotypes. A previous report[19] has suggested *RYR1* genotype-dependent influences on IVCT responses, but insufficient patients were available to examine confounders such as sex, age, central core morphology, and muscle specimen characteristics. Genetic background represents another potential source of variability in IVCT response and CK concentration that might confound such studies. Patients from ethnic minorities represent ~4% of our study population with the majority of these being of Indian or Pakistani descent: other groups represented are Black African, Black Caribbean, and Chinese. However, genetic stratification can occur within ethnic groups and we therefore elected to correct for genetic background by including family background as a random effect in our mixed model analysis.

Our results provide strong evidence that different *RYR1* variants are associated with significant differences in IVCT and CK phenotypes; more specifically, the variants p.R163C, p.R2163H, p.R2435H, and p.T4826I were significantly associated with a more severe form of each quantitative phenotype than p.G2434R. These variants have previously been functionally characterized in expression systems[6 20] showing a greater sensitivity to chemically evoked intracellular Ca^{2+} release than cells expressing wild-type RyR1. The variant p.S1728F, on the

Table 6 Analysis of the relationships between MH-related phenotypes and *RYR1* variant characteristics. χ^2 and *P*-values from mixed model analyses, with both variant and family included as random effects, and sex, age, and muscle specimen characteristics included as covariates. Figures in bold represent statistically significant results of $P<0.05$. *MH/CCD mutations were defined as those being known to be associated with both the MH and the CCD phenotypes, p.R163C, p.R614C, p.R2163H, p.R2435H, and p.V4849I. †Conserved site defined as a site where an amino acid is conserved within all species in the alignment or mammals only. ‡RYR isoform conservation is defined as a site where an amino acid is conserved in all three RYR isoforms

Explanatory variable	Dependent variable				
	Static caffeine tension	Static halothane tension	Dynamic halothane tension	Static ryanodine time	Creatine kinase
MH/CCD *vs* non-MH/CCD variant*	$\chi^2_{(1)}$=0.67; *P*=0.413	$\chi^2_{(1)}$=1.81; *P*=0.179	$\chi^2_{(1)}$=5.28; ***P*=0.022**	$\chi^2_{(1)}$=5.14; ***P*=0.023**	$\chi^2_{(1)}$=3.57; *P*=0.058
Conserved *vs* non-conserved site†	$\chi^2_{(1)}$=5.78; ***P*=0.016**	$\chi^2_{(1)}$=0.19; *P*=0.665	$\chi^2_{(1)}$=2.33; *P*=0.127	$\chi^2_{(1)}$=5.48; ***P*=0.019**	$\chi^2_{(1)}$=1.78; *P*=0.182
Conserved *vs* non-conserved site across RYR isoforms‡	$\chi^2_{(1)}$=2.31; *P*=0.192	$\chi^2_{(1)}$=3.59; *P*=0.058	$\chi^2_{(1)}$=3.22; *P*=0.073	$\chi^2_{(1)}$=2.70; *P*=0.100	$\chi^2_{(1)}$=1.27; *P*=0.260

other hand, was consistently associated with significantly weaker IVCT phenotypes in comparison with p.G2434R, suggesting a lesser effect on channel function.

Further variants were associated with significant differences in one or more, but not all, quantitative phenotypes in comparison with p.G2434R. In some cases, this may reflect a lack of power for comparisons involving variants carried by only a few individuals, for example, p.G3990V and p.D3986E. With other variants, however, there appear to be genuine differences between the different phenotypes in comparison with p.G2434R. For example, p.G341R is associated with similar halothane responses to p.G2434R but significantly more severe caffeine and ryanodine responses, whereas p.T2206M is associated with a significantly shorter ryanodine onset time than p.G2434R but similar caffeine and halothane responses: it should be noted that the differences are significant after correcting for multiple comparisons. These findings suggest that caffeine, ryanodine, and halothane have different mechanisms of action at the protein level and may inform future studies of these mechanisms.

Muscle specimen length, and also weight in the case of ryanodine response time, was found to play a significant role in the variation observed in the IVCT response. The extent of the pre-drug electrically evoked twitch response (indicating muscle viability) also significantly contributes to the IVCT response for all phenotypes, except dynamic halothane. The data presented here suggest that males experience a more severe response phenotype with dynamic halothane but not for any other IVCT phenotype or CK. It is important to note that the effects of these potential confounders have been accounted for in the analysis.

From a clinical perspective, the genetic risk of developing MH has previously been considered as an 'all-or-none' phenomenon, with the assumption that heterogeneity in clinical presentations[8] was a result of 'environmental' factors. One difficulty in challenging this dogma is establishing a means of quantifying the clinical MH reaction. A previously described classification system[21] and the MH Clinical Grading Scale[22] have both proved useful in assessing how likely a clinical reaction is to be a true MH reaction. However, the grade or class attributed using these tools is heavily dependent on the delay in diagnosis and adequacy of treatment implemented after diagnosis.[8] They are also greatly influenced by missing clinical data. The concept we propose is that the rate of development of the clinical features of MH is dependent on the rate of accumulation of skeletal muscle intracellular Ca^{2+}, which in turn is dependent on the severity of the causative genetic defect. The clinical response time, as defined and recorded in this study, uses endpoints that can be identified in retrospect to have occurred before any treatment interventions were made. It does assume that these signs were caused by the MH response, but we think this is reasonable in the context of subsequent clinical diagnosis, confirmation by IVCT, and the finding of an *RYR1* variant.

Even with a data set as large as ours, however, interrogation of the influence of different *RYR1* variants on the clinical onset time lacked sufficient power to draw meaningful conclusions. We therefore examined the relationship between clinical response time and each quantitative phenotype to explore the suitability of the latter as surrogates of the clinical reaction. We demonstrated significant correlations between three out of four quantitative IVCT phenotypes and CK concentration and the clinical response time and these findings were sustained after accounting for potential confounding variables. Our findings suggest that differences in IVCT responses and CK concentration are clinically relevant quantitative phenotypes in the study of MH.

These results provide the most tangible evidence to date that genetic differences explain, at least in part, variability in clinical MH responses, albeit inferred from data using a quantitative surrogate marker. It is possible that variants associated with a less severe IVCT phenotype are more likely to be associated with reduced clinical penetrance: unfortunately, there is insufficient statistical power within our data set to test this hypothesis. This may, however, explain why some familial *RYR1* variants are consistently found in ~5% MHN individuals from families with the familial *RYR1* variant.[23] Put together with validated evidence for multiple interacting gene products conferring MH status,[24 25] these and the current findings make the prospect of a threshold model for MH susceptibility quite attractive. In such a model, the 'stronger' *RYR1* variants

would be sufficient alone to cause MH susceptibility, but the clinical risk associated with 'weaker' *RYR1* variants would be dependent on other modifying genetic factors. From a clinical safety perspective, however, all *RYR1* variants associated with abnormal IVCT responses in MH families should at present be considered potential risk factors for the development of MH under anaesthesia.

Our analyses demonstrate that some variants are associated with average IVCT responses only just above the abnormal threshold values and some are associated with consistently different responses to halothane and caffeine. These findings argue against previous assertions that the IVCT threshold values are too low, that the MH equivocal diagnosis is of limited value, and that caffeine responses are inherently better indicators of the presence of *RYR1* variants than halothane responses.[26]

We observed an association between CK concentration and *RYR1* variant, and this supports previous reports of higher mean CK concentrations observed with MH-susceptible individuals.[27 28] These findings suggest that some *RYR1* variants are more likely to be associated with increased Ca^{2+} cycling under physiological conditions (as opposed to only upon exposure to triggering anaesthetics). It is interesting that p.R163C, which we found to be associated with high CK concentrations, has been found in patients experiencing exertional rhabdomyolysis[29] and in a fatal case of exertional heat stroke.[30]

CK concentrations have limited diagnostic value in MH, however, because of the large degree of overlap in CK concentrations between MH and normal populations. There is further lack of specificity in using CK concentration as a predictor because there are many other causes of a raised CK concentration. It is, however, patients with variants associated with increased CK concentration who may be more likely to present as cases of idiopathic hyperCKaemia.[11] Such patients are difficult to manage and are often refused anaesthesia for fear of MH. Screening for 'high CK' *RYR1* variants in such patients, or conducting familial studies of CK concentrations, may represent a cost-effective approach to identifying MH risk in patients with persistently raised CK.

RYR1 variant characteristics

In each of three separate secondary analyses, we divided *a priori* the *RYR1* variants into two groups according to the presence or absence of a shared characteristic and compared the collective quantitative phenotypes of the groups. Dirksen and Avila[31] have described distinct effects of *RYR1* variants associated with MH compared with those associated with CCD, and it has been proposed that those associated with the coincidence of both conditions have different properties from those associated with MH alone.[10] Our preliminary work[9] suggested that variants associated with the coincidence of CCD and MH susceptibility produced MH phenotypes at the more severe end of the spectrum of IVCT responses. In the current study, variants described in relation to both MH and CCD were associated with significantly more severe dynamic halothane (P=0.022) and ryanodine (P=0.023), but not the other, phenotypes in comparison with the group of variants that have not been associated with CCD. Individually, p.R163C, p.R2163H, and p.R2435H showed a more severe IVCT phenotype compared with p.G2434R, but p.R614C and p.V4849I were not significantly different in their IVCT responses. Furthermore, p.T4826I, which has not been observed in CCD patients, displayed a more severe IVCT phenotype. These data imply that the severity of the MH phenotype is not exclusively correlated with those variants described with both MH and CCD and that factors other than the sensitivity to chemically evoked intracellular Ca^{2+} release determine the potential for a

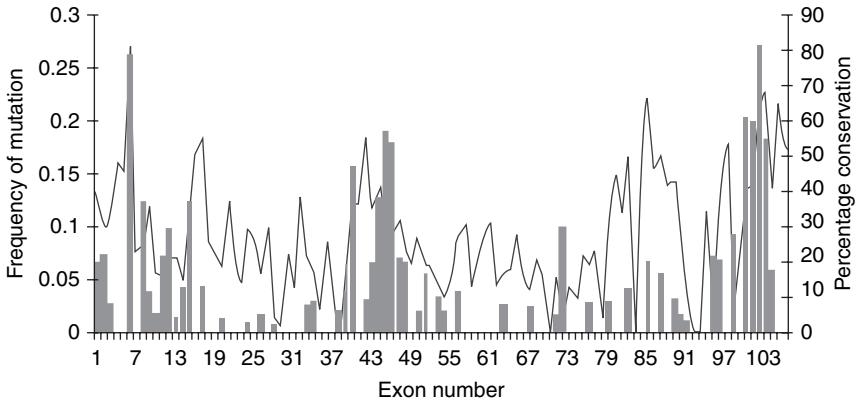

Fig 2 The global distribution of *RYR1* variants together with the percentage conservation for each exon. The total number of variants per exon was calculated from Robinson and colleagues,[5] and divided by the number of amino-acids per exon to give a frequency of variants per exon, and is represented by the grey bars. The percentage of evolutionarily conserved sites within each exon was calculated from the sequence alignment data using all available species alignments [species used were *H. sapiens* (P21817), *S. scrofa* (P16960), *M. musculus* (Q80X16), *O. cuniculus* (P11716), *D. melanogaster* (Q24498), *C. elegans* (P91905), *A. gambiae* (Q7PJQ9), *R. catesbeiana* (Q91313), *M. nigricans* (O13054), and *H. pulcherrimus* (Q8TA74)], and is shown with the continuous line.

variant to be associated with the CCD, in addition to the MH, phenotype.

The two other groupings were made according to conservation of the amino acid position; first, in all species or in mammals only and, secondly, in all human RyR isoforms or not. The conservation of a site within a protein over evolutionary time suggests that the position may be of functional importance. Analysis of conservation data provides some information about the nature of the protein, and sites of potential functional relevance. The data presented here provide evidence that altering a site that is conserved in all species studied is correlated with stronger caffeine responses (P=0.016) and shorter ryanodine response times (P=0.023), suggesting a functional importance to these evolutionarily conserved sites. Unfortunately, only three variants were not conserved in the three human RyR isoforms and no conclusions can be drawn from these data.

The majority of *RYR1* variants have been described in three clusters corresponding to the following regions of the RyR1 protein: N-terminal (Cys35-Arg614, MH region 1), central (Asp2129-Arg2458, MH region 2), and C-terminal (Ile3916-Ala4942, MH region 3). The C-terminal region forms the pore of the Ca^{2+}-release channel and contains the majority of variants associated with CCD.[10] It is interesting that these regions contain a high frequency of evolutionarily conserved sites (Fig. 2). There are, however, other regions of *RYR1* with high conservation frequency that contain fewer identified *RYR1* variants. This may be the result of screening bias as evidenced by the results of the full *RYR1* cDNA screening presented here: this increased the number of exons with mis-sense changes from 21 to 53. However, the potential functional importance of the 'hot-spot' regions cannot be discounted, especially as the two variants included in the current phenotype–genotype analysis that were not from these regions, p.S1728F and p.E3104K were both associated with weaker phenotypes.

The clustering found in *RYR1* has also influenced variant screening strategies used in the screening of the cardiac *RYR2* isoform, implicated in catecholaminergic polymorphic ventricular tachycardia and arrhythmogenic right ventricular cardiomyopathy/dysplasia type 2.[32 33] Furthermore, the clustering of mutations in *RYR1* and *RYR2* has led to the hypothesis that the mutation 'hot-spot' regions contain domains that interact with domains in the other mutation 'hot-spot' regions to regulate channel function.[34–36]

Consistent with interacting domains, the *RYR1* variants in the present study having a significantly (P<0.01) greater response to caffeine (p.R163C, p.G341R, p.R2163H, p.R2435H, p.R2454H, p.D3986E, p.G3990V, and p.T4826I) than p.G2434R are distributed throughout the gene. The spread of these variants does suggest a number of different sites within *RYR1* that are potentially involved in caffeine sensitivity, reported here to be correlated with the nature of the MH reaction. Unfortunately, there is a significant proportion of the RyR1 structure and topology still unknown or yet to be fully corroborated.[37]

In conclusion, we have provided evidence that different *RYR1* variants vary in the severity of IVCT and CK concentration MH phenotype that they are associated with. These phenotypes, in turn, are associated with the speed of onset of clinical MH episodes. Our data are consistent with a 'threshold' model of MH susceptibility in which the inheritance of certain ('weaker') RYR1 variants may be insufficient to confer clinical MH risk without the co-inheritance of other genetic factors.

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