The role of *CACNA1S* in predisposition to malignant hyperthermia

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ABSTRACT

Malignant hyperthermia (MH) is an inherited pharmacogenetic disorder of skeletal muscle, characterised by an elevated calcium release from the skeletal muscle sarcoplasmic reticulum. The dihydropyridine receptor (DHPR) plays an essential role in excitation-contraction coupling and calcium homeostasis in skeletal muscle. This study focuses on the gene CACNA1S which encodes the α1 subunit of the DHPR, in order to establish whether CACNA1S plays a major role in MH susceptibility in the UK. We investigate the CACNA1S locus in detail in 50 MH patients, the largest to date, to identify novel variants that may predispose to disease, and also to characterise the haplotype structure across CACNA1S. We present CACNA1S cDNA sequencing data from 50 MH patients in whom RYR1 mutations had been excluded, and subsequent mutation screening analysis. Furthermore we present haplotype analysis of unphased CACNA1S SNPs to (1) assess CACNA1S haplotype frequency differences between susceptible MH cases and a European control group and (2) analyse population-based association via clustering of CACNA1S haplotypes based on disease risk. The study identified a single potentially pathogenic change in CACNA1S (p.Arg174Trp), and highlights that the haplotype structure across CACNA1S is diverse, with a high degree of variability.
INTRODUCTION

Malignant hyperthermia (MH) is an inherited disorder of skeletal muscle, which predisposes to an increased release of calcium into the myoplasm under certain pharmacological conditions. Inhalational anaesthetics and the muscle relaxant suxamethonium can trigger an MH crisis and lead to acceleration of muscle metabolism and contractile activity generating heat and leading to hypoxaemia, metabolic acidosis, rhabdomyolysis and a rapid rise in body temperature. This condition can be potentially fatal if not recognised and treated promptly.

Biochemical studies have shown that an MH crisis is due to an abnormal cellular calcium homeostasis within the skeletal muscle [1]. Within skeletal muscle the sarcoplasmic reticulum (SR) controls the process of Ca\(^{2+}\) release, playing a major role in the process of excitation-contraction (E-C) coupling. During E-C coupling depolarisation of the sarcolemma initiates a conformational change in the voltage-gated Ca\(^{2+}\) channel (dihydropyridine receptor (DHPR)) subsequently activating the Ca\(^{2+}\) release channel (Ryanodine receptor (RyR1)) to release Ca\(^{2+}\) from the SR [2]. During an MH crisis an elevated rate of cellular Ca\(^{2+}\) release from the SR is observed due, in part, to a reduced activation and increased deactivation threshold of the RyR1 [3], or from uncoupling of the DHPR-RyR1 interaction [4].

Genetic analyses have demonstrated that MH susceptibility exhibits locus heterogeneity, with significant observations for linkage to chromosome 1q [5,6] and 19q [7,8]. The locus on chromosome 19q has been identified as the gene encoding the skeletal muscle ryanodine receptor (RYR1) [8], and that on chromosome 1q as the gene encoding the \(\alpha_1\) subunit of the DHPR (CACNA1S) [5]. There is a finely
balanced interaction between the gene products of *RYR1* and *CACNA1S*, which are only beginning to be understood with alterations in both gene products affecting E-C coupling and modifying Ca\(^{2+}\) regulation [3,4].

Much research into MH susceptibility has been focused on the *RYR1* locus and it is recognised that *RYR1* plays a major role in susceptibility to MH. There are now over 178 mis-sense mutations described across *RYR1* that co-segregate with MH susceptibility, 29 of which have been functionally characterised and are used diagnostically [reviewed in 9]. In the UK, *RYR1* plays a part in MH susceptibility in over 70% [394/554] of UK pedigrees. Considerably less however is known about *CACNA1S*. Previous studies have demonstrated linkage to chromosome 1q within MH families that show *RYR1* exclusion [10], but to date there is only a single mis-sense change (p.Arg1086His) described in association with MH [5]. This change was first detected in a single extended French family in 12 individuals all diagnosed as susceptible to MH, and absent from the 6 individuals diagnosed as normal [5]. In a North American study of 98 independent MH samples this change was also identified in a single family [11], in 2 from the 5 MH diagnosed individuals. p.Arg1086His was not detected in 100 independent normal French chromosomes [5], nor in 150 unrelated North America normal samples [11]. Interestingly, this change has further been described alongside an *RYR1* alteration [p.Pro4973Leu] in a single individual [12], where the rest of the family diagnosed as MHS were accounted for by either the *RYR1* change [three individuals] or the *CACNA1S* change [two individuals], suggesting a potentially more complex means of MH susceptibility involving multiple gene products.
The aim of this study is to investigate the CACNA1S locus in detail and to determine whether CACNA1S may play a major role in MH susceptibility in the UK. As targeted sequencing for RYR1 has led to potential bias in mutation detection, we have sequenced the full cDNA transcript of CACNA1S for novel changes in 50 independent MH families. We report here the findings of this sequencing and subsequent mutation screening. Furthermore characterisation of the haplotype structure across CACNA1S was investigated. We present analysis using unphased CACNA1S SNP data directly to (1) assess CACNA1S haplotype frequency differences between MH susceptible cases and a population control group and (2) to analyse population-based association via clustering of CACNA1S haplotypes based on disease risk.

MATERIALS AND METHODS

In-vitro contracture testing

There is a well defined and standardised protocol for the laboratory confirmation of suspected MH cases and testing of family members. The in vitro contracture test (IVCT), involves exposure of skeletal muscle biopsy specimens to incremental concentrations of halothane or caffeine in an irrigated tissue bath, and the subsequent measurement of muscle contracture in response to the applied stimulants. All individuals were phenotyped by the IVCT according to the European MH Group guidelines (www.emhg.org) at the MH Investigation Unit at St James’s Hospital, Leeds, UK. The European protocol assigns the patient to one of 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 deemed to represent clinical susceptibility to MH.
Samples

This study utilises the largest worldwide resource of genotyped MH samples from patients phenotyped in a single diagnostic centre. CACNA1S sequencing was performed on 50 independent UK MH susceptible samples. These samples comprise 30 MHS samples and 20 MHE (responding to halothane but not caffeine) samples; 14 of these MHE samples are from probands who suffered a clinical reaction. The remaining 6 MHE samples are the only available representative from these individual families. All 50 of these samples have had the RYR1 cDNA transcript sequenced and have had no variants detected. There are 33 males and 21 females in the cohort and the ages range from 10-75 years, with a mean of 33 years. The IVCT data for the 50 patients show a median (range) contracture of 0.6 g (0.2-4.7 g) at 2% halothane and 0.2 g (0 – 3.6 g) at 2 mM caffeine.

Mutation screening was performed on an additional 410 independent UK MH patients to give a total of 460 independent MH families. Of the 460 independent MH patients, 340 (74%) have an RYR1 mis-sense change assigned, of these 298 (65%) co-segregate with disease, 226 (49%) have a functionally characterised RYR1 change and there are 8 instances of compound heterozygosity with two different RYR1 changes [one instance of c.1021G>A/c.7025A>G; c.7063C>T/c.7025A>G; c.7036G>A/c.14817C>A; c.4024A>G/c.4088C>T; c.5441T>A/c.7528T>C; c.10616G>A/c.14210G>A; and two instances of 7300G>A/7373G>A;]. 100 independent MHN samples were also screened.

CACNA1S haplotype analysis was performed on 460 independent UK MH patients, the same as used for mutation screening assays. Population control samples (n=480)
for the haplotype analysis were obtained from a DNA panel of Human Random Controls manufactured by the European Collection of Cell Cultures (ECACC). The DNA is derived from peripheral blood lymphocytes of UK Caucasian donors with informed consent.

**CACNA1S sequencing**

CACNA1S cDNA sequencing was performed on 50 independent MH susceptible individuals, who did not have an RYR1 mis-sense change after having previously been sequenced for the RYR1 cDNA transcript. cDNA prepared from total RNA isolated from muscle biopsy specimens was used to sequence the ~6.16kb CACNA1S cDNA, using 12 overlapping fragments of approximately 700bp in length, read in both the forward and reverse direction and analysed on an ABI3730.

**Mutation analysis**

A novel p.Arg174Trp/c.520C>T change in exon 4 causes a loss of an MspI site, thus further screening analysis was performed on genomic DNA using the forward primer 5’-CTC AAG CAT GGA CAG GAC AC-3’, and reverse primer 5’-AG G AAG GGA GAG GAG AAA GG-3’ to generated an amplicon of 279bp. In the normal (c.520*C) this is cleaved to produce 3 fragments of 49bp, 67bp and 163bp. Cleavage at one of the sites fails to occur in the presence of the mutated allele, c.520*T, thus generating 2 fragments of 116bp and 163bp in length.

The previously described CACNA1S mutation p.Arg1086His/c.3257G>A in exon 26 was screened for in the full cohort of independent UK MH patients using an assay developed in-house as follows: forward 5’ ATG CAC CCT ACC CTA TCT CC-3’,
and reverse 5’-GGA GCA GGG AGC CTA GTT AC-3’ primers generate an
amplicon of 998bp in length. In the normal (c.3257*G) this is cleaved by HhaI to
produce 3 fragments of 362bp, 316bp and 313bp. Cleavage at one of the sites fails to
occur in the presence of the mutated allele, c.3257*A, to generate 2 fragments of
629bp and 362bp in length.

**Haplotype analysis**

**Haplotype construction**

Focusing at the genomic DNA level there are >175 SNPs described across the 73kb
CACNA1S gene; predominantly listed in internet databases sources, in particular the
CEPH population of the Hap-Map project ([www.hapmap.org](http://www.hapmap.org)), and a further 3
identified through in-house CACNA1S sequencing. When concentrating on SNPs
with a minor allele frequency greater than 0.05 the total number of described SNPs
spanning the gene is reduced to 115, 16 of which are located in exons. Using the
Tagger software on Haploview we selected eight informative SNPs to span
CACNA1S. The final list of SNPs chosen is detailed in table 1.

All SNPs were genotyped using Taqman methodology. For all SNPs there is an ABI-
assay-on-demand available (table 1). All allelic discrimination assays were carried
out on an ABI 7900 according to the manufacturer’s instructions. Linkage
disequilibrium between the SNPs was calculated using the Haploview software [13].

**Statistical analysis**

The program PHASE V2 was used as a method for reconstructing haplotypes from
the unphased CACNA1S genotype data and to perform case control permutation tests
between MHS samples and population control samples [14,15]. PHASE calculates the posterior probability distribution of haplotypes through a Bayesian statistical approach, combining a specified prior for a statistical model for population genetics and likelihood information. The program has a function for case control permutation testing. This tests the null hypothesis that haplotype frequencies are the same in cases and controls, versus the alternative hypothesis that haplotype frequencies are different between the two groups.

The program GENEBPM, a program designed for use with candidate genes, tests for association of disease with causal variants at an unseen functional polymorphism [16,17]. This program makes use of the expectation that a pair of haplotypes carrying the same disease mutation are more likely to share a more recent common ancestry than a random pair of haplotypes in the population and thus are more likely to be similar to each other in terms of their allelic make-up at flanking markers. Furthermore, output of the algorithm can be used to ascertain clusters of haplotypes that are associated with specific causative variants, and to estimate the odds of disease for these unobserved alleles.

RESULTS

**CACNA1S sequencing**

Full cDNA sequencing identified non-synonymous changes in CACNA1S in 12 individuals, 24% of the MH cases. Sequence changes lead to modifications of amino acids at positions 69 (n=4), 174 (n=1), 258 (n=4), 458 (n=13), 606 (n=1), 1541 (n=4) and 1660 (n=5), all present as heterozygous changes, except the change at position 458 which was also observed in both homozygous forms. However, the changes at
positions 1541 and 1660 are previously described polymorphisms (rs3850625 and rs13374149 respectively). The change at position 458 has also been described as polymorphic [5] and indeed was found to be highly variable in our cohort with a heterozygosity of 0.425. The other substitutions detected were in codons determining amino acids that are conserved in rabbit, cat, mouse and zebrafish [NCBI reference sequences NP_001095190, NP_001033694, NP_001074492.1, NP_999891.1 respectively] and therefore potentially deleterious mutations rather than infrequent polymorphisms. However, the first change p.Ala69Gly, whilst being detected in 4 MH susceptible individuals, was also detected in 7 from 100 MHN samples, and is therefore likely to be a polymorphism. Furthermore, the changes p.Gly258Asp and p.Ser606Asn, whilst not being detected in 100 MHN controls, were observed to be frequently discordant with MH status in families and can be added to the growing number of non-synonymous changes reported across CACNA1S, over half (10) of which are likely to be polymorphisms [see table 2].

The final mis-sense variation that was detected, p.Arg174Trp, was found in an MHS sample, was concordant with disease within the family and also not detected in 100 MHN control samples. The mother of the proband was diagnosed MHS through the IVCT, and also had the p.Arg174Trp alteration. A sibling of the proband, diagnosed normal through the IVCT, did not have the p.Arg174Trp change. The MH proband, in whom the p.Arg174Trp was detected, developed intense and prolonged spasm of the jaw muscles after administration of the inhalation anaesthetic halothane and the muscle relaxant suxamethonium Post-operatively there was severe muscle stiffness that persisted for 2 weeks and a peak serum creatine kinase concentration of 15,500 IU/L (normal < 210 IU/L).
The cDNA sequencing further identified 3 novel silent changes in the CACNA1S gene. These were located in 2 different exons; p.Leu766/c.2296C>T minor allele frequency of 0.01 and a heterozygosity of 0.02, and p.Ile781/c.2343C>T, with a minor allele frequency of 0.031 and a heterozygosity of 0.06 were located in exon 17, and p.Pro1622/c.4866C>T with a minor allele frequency of 0.208 and a heterozygosity of 0.33 was located in exon 40. There is no significant linkage disequilibrium detected between the markers, with $r^2=0$ between 4866*C and both 2296*C and 2343*C, and an $r^2=0.32$ between the neighbouring markers 2296*C and 2343*C. Linkage disequilibrium was also not detected between these markers and their adjacent markers across CACNA1S, ie between 4866*C with either rs3850625*C or rs13374149*G and nor between 2296*C and 2343*C with rs7415038*T or rs1684767*C.

**Mutation screening**

The p.Arg174Trp change was identified in a single family showing full concordance with disease status and not identified in 100 normal controls. Accordingly we screened for the presence of this site in the full UK cohort of 410 independent MH families. The p.Arg174Trp change was not detected in any other UK family.

The previously described CACNA1S mutation p.Arg1086His in exon 26 was screened for in the 460 independent UK MH patients. This change was not detected in any UK MH family, nor the 100 MHN controls.

**Haplotype analysis**
A total of 460 independent UK MH patients and 480 Caucasian population controls were typed for all eight CACNA1S SNP markers. These 8 SNPs were used to reconstruct haplotypes from the unphased data using PHASE and GENE BPM. From GENE BPM there were a total of 23 haplotypes with an estimated population frequency ≥0.01/1%, and these are detailed in table 3, along with a breakdown of the haplotype frequencies for each study group (MHS, PC and also the subset of 50 samples that were sequenced for CACNA1S) calculated through PHASE. The single SNPs were tested for association with disease using a Spearman rank correlation between MHS samples and PC. There was no evidence for significant associations with any of the markers except p.Ile199 where p=0.014.

Case control permutation testing was performed between MHS samples and the population control samples to test for differences in CACNA1S haplotype frequencies using the program PHASE. There was a small but significant difference observed with this comparison (p=0.02), providing evidence for association between MH and CACNA1S. However, for the same comparison using the GENE BPM program to analysis haplotype relative risk of disease, there was no categorical evidence of CACNA1S haplotype association with MH (posterior probability \( \hat{\rho} = 0.46 \)).

To illustrate the posterior similarities between haplotypes, in terms of their risk of carrying causal variants and allelic make-up, a dendrogram can be constructed. Figure 1 presents a dendrogram of the 23 CACNA1S haplotypes with estimated relative frequency ≥1% from the analysis of MHS cases versus population controls. These 23 haplotypes are coded according to their relative frequency, where 1 represents the most frequent haplotype, and 23 the least frequent. The dendrogram
shows considerable posterior similarity between haplotypes and demonstrates no apparent clustering of haplotypes, suggesting that there is no clear high risk disease variant. Additionally, the haplotype analysis reveals that there are a lot of relatively rare haplotypes (there are only three haplotypes with a frequency >5% in population controls), suggestive of an elevated degree of haplotype diversity potentially resulting from a high rate of recombination across the locus and a low level of linkage disequilibrium.

To exclude any influence of *RYR1* on the analysis a case control permutation test in PHASE was also performed between the 480 population control samples and the 50 samples that have been cDNA sequenced for *CACNA1S*. There was no overall significant difference in *CACNA1S* haplotype frequency observed between these groups. The haplotypes H5 and H14 were observed at a noticeably higher frequency in the 50 sequenced samples (0.099 and 0.058 respectively) than the population controls (0.043 and 0.029 respectively) (see table 3), however given that the haplotype frequencies are relatively small these observations are unlikely to be significant. Further analysis with GENEBPM also provided no categorical evidence of *CACNA1S* haplotype association with MH (posterior probability \( \hat{\rho} = 0.415 \)), suggesting that *CACNA1S* does not play a major role in MH susceptibility, however due to the small number in the cDNA sequenced group this comparison may lack power.

Furthermore to investigate whether there were any differences in *CACNA1S* haplotype frequencies between MH phenotypes, an additional case control permutation test was performed on the subset of 50 samples that underwent cDNA sequencing between the
MHS (n=30) and MHE (n=20) samples using all 8 SNPs. There was no significant difference in \(\text{CACNA1S}\) haplotype frequency observed between the MHS and MHE samples (p=0.27), again due to the small numbers in each group this comparison may lack power.

**DISCUSSION**

As more is being understood about the nature of susceptibility to MH it is becoming increasingly apparent that it is complex, and cannot always be simply described as autosomal dominant. There is evidence for variation in clinical severity, and IVCT phenotype, resulting from the same mis-sense change in \(\text{RYRI}\),\(^{18,19}\) reports of compound heterozygotes in \(\text{RYRI}\) [12, 20, unpublished UK observations], and an individual with mutations in both \(\text{RYRI}\) and \(\text{CACNA1S}\) [12]. Furthermore, we have previously demonstrated, using transmission disequilibrium testing, that multiple interacting gene products affect susceptibility to MH [10,21]. Even within families that showed linkage to \(\text{RYRI}\) there was evidence provided for linkage to other loci elsewhere in the genome [10].

This study focused on \(\text{CACNA1S}\) encoding the \(\alpha_1\) subunit of the DHPR. In the largest study to date, of 50 MH patients, we identified a single, potentially pathogenic, variant p.Arg174Trp. The p.Arg174Trp change is situated at a site that is conserved in rabbit, cat, mouse, and zebrafish and causes a change in the charge of the amino acid from basic to non-polar. The amino acid in question lies in the S4 segment domain of the DHPR thought to function as a voltage sensor, thus a change in charge may alter the voltage sensor mechanism and consequently disrupt the cellular calcium

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homoeostasis. Further functional work to support these observations would be valuable.

This work also identified two other variants (p.Gly258Asp, p.Ser606Asn) thought to be polymorphic as they do not show disease concordancy, but they were not present in control chromosomes. Whilst it is likely that they are indeed polymorphisms, the fact that they lie in conserved regions of the protein and cause a change in the polarity of amino acid substituted suggests otherwise and there is the potential that they could play a minor role, or have a modifier effect, on disease phenotype. Since there is evidence that MH may not necessarily be a simple single gene disorder, there is the possibility that both of these changes are present together with an additional major change and in some instances account for discordancy with disease. i.e. these mutations may be necessary, but not sufficient, to cause MH susceptibility in particular individuals.

Comparison of CACNA1S haplotype frequencies between susceptible cases and UK Caucasian population controls identified no significant haplotype frequency differences. Even given that this is the largest standardised and genotyped MH database worldwide, there are a limited number of MH patients, which could reduce the power to detect a significant association between CACNA1S haplotype with MH. However, the haplotype analysis does provide some evidence for an elevated haplotype diversity, potentially resulting from the high rate of recombination observed across the locus and a low level of linkage disequilibrium detected, as seen in the present study and consistent with that observed in the HapMap project. Couple this observation with the, now growing, number of reported non-pathogenic non-
synonymous changes described across CACNA1S, then it is possible that this locus can tolerate a high degree of variability. This has implications, not only in fully comprehending the E-C coupling of skeletal muscle, but also that in cardiac muscle.

Our data suggest that, whilst CACNA1S may play a role in MH manifestation in the UK, it is not a major locus, thereby suggesting that there are other loci with importance in MH susceptibility. As well as the reported linkage to chromosome 1q and 19q there are alternative loci proposed on chromosomes 7q [22] and 17q [23], however no contributory mutations have been identified in these regions. It is highly probable that any novel genes for MH susceptibility will play a minor role. An alternative method to identify genes responsible for MH, could be to take a candidate gene approach and focus on genes whose products are directly involved with E-C coupling and Ca$^{2+}$ regulation, for example the other subunits of the DHPR, calmodulin$^{24}$ and JP-45 [25].

We have previously proposed that several independent genes can influence the MH phenotype [10,21]. Here we present evidence for a novel variant in CACNA1S which could have the potential to directly influence MH susceptibility. There was also a possible indirect or modifier effect of CACNA1S in a small number of families, likely to cause MH in combination with another, as yet unknown, locus. The evidence for the existence of multiple independent loci that influence MH susceptibility is now increasing and the disorder appears to be more complex than previously thought. To fully comprehend MH and all the gene product interactions we need to identify and characterise all the multiple loci involved.
REFERENCES


Figure 1: Dendrogram of the 23 marker haplotypes with estimated population frequency $\geq 0.01$ (1%), to demonstrate similarities between haplotypes in terms of disease risk and marker sharing created from the output of a single run of the MCMC algorithm in GENE BPM. Haplotypes are coded according to their relative frequency, thus haplotype 1 is the most common haplotype, and haplotype 23 the least.
Additional files provided with this submission:

Additional file 1: table 1.doc, 36K
http://www.biomedcentral.com/imedia/8171202542716429/supp1.doc
Additional file 2: table 2.doc, 49K
http://www.biomedcentral.com/imedia/1654032928271642/supp2.doc
Additional file 3: table 3.doc, 55K
http://www.biomedcentral.com/imedia/9904536152716429/supp3.doc