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TRANSLATIONAL RESEARCH

Sequence capture and massively parallel sequencing to detect mutations associated with malignant hyperthermia

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Editor's key points

- In up to half of families with malignant hyperthermia (MH) susceptibility, no causative genetic mutation has been identified.
- Genomic DNA capture and next-generation sequencing was used to screen for multiple candidate genes in two patients.
- Possible causative mutations were identified for one patient.
- The efficacy of the techniques described will be useful in the future.

Background. Malignant hyperthermia (MH) is a potentially fatal pharmacogenetic disorder in which intracellular calcium homeostasis in the skeletal muscle of susceptible individuals is disrupted upon exposure to halogenated anaesthetics. While MH is linked to the ryanodine receptor (*RYR1*) on chromosome 19 and the α1S subunit of the voltage-dependent L-type calcium channel (*CACNA1S*) on chromosome 1, mutations have been found in only 50–70% of patients, and subsequently, there is a need for a more powerful screening tool.

Methods. Genomic DNA capture and next-generation sequencing was used to screen 32 genes involved in excitation–contraction coupling, skeletal muscle calcium homeostasis, or immune response in two MH patients. Lymphoblastoid cell lines were used to functionally characterize candidate *RYR1* mutations in one family.

Results. Sequence analysis revealed two putative causative mutations in *RYR1* in one patient. Segregation analysis and functional analysis support a causative role of the detected variants. The amount of Ca^{2+} released after stimulation with 4-chloro-*m*-cresol from B lymphocytes of the MH-susceptible patients in the family was significantly greater compared with that of Ca^{2+} released from cells of an MH-negative family member. In the other patient, no causative mutations were identified in the 32 genes screened.

Conclusions. In this study, we successfully demonstrate the use of genomic DNA capture and next-generation sequencing for identification of putative mutations causing MH. We also suggest that whole exome sequencing may be necessary to identify MH causing mutations in patients where no mutations in *RYR1* and *CACNA1S* have been identified thus far.

Keywords: genetic factors; malignant hyperthermia; measurement techniques, ion, calcium; muscle skeletal; screening

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Malignant hyperthermia (MH; OMIM# 145600) is a potentially lethal autosomal-dominant pharmacogenetic disorder. The prevalence of MH has been estimated between 1:2000[1] and 1:100 000[2] and is triggered in susceptible individuals by exposure to volatile anaesthetics or depolarizing neuromuscular blocking agents during general anaesthesia. During an MH episode, the myoplasmic calcium level increases rapidly due to increased flux of calcium from the sarcoplasmic reticulum (SR) to the cytosol. This results in a hypermetabolic state characterized by hyperthermia, muscle rigidity, tachycardia, hypoxaemia, and metabolic acidosis, which if untreated, can lead to death.

In excitation–contraction (EC) coupling, the dihydropyridine receptor (DHPR, L-type voltage-dependent calcium channel) located at the transverse tubules undergoes conformational changes upon depolarization of the plasma membrane in skeletal muscle and therefore activates the ryanodine receptor which in turn releases calcium from the SR into the sarcoplasm, leading to muscle contraction and other metabolic activities.[3] The primary locus for MH has been identified as the *RYR1* gene on 19q13.1 encoding the skeletal muscle form of the ryanodine receptor, which is the major skeletal muscle calcium-release channel. In MH, mutations in the *RYR1* gene result in an increased release of calcium from the SR into the sarcoplasm.

In most studies, missense amino acid changes in the ryanodine receptor have been identified in 50–70% of MH families.[4 5] Less than 1% of MH cases can be attributed to mutations in the α1S subunit of the voltage-dependent L-type calcium channel (*CACNA1S*).[6 7] Another potential locus is calsequestrin 1 (*CASQ1*),[8] although no causative mutation has yet been identified in humans. Therefore, in 30–

50% of MH-susceptible (MHS) families, mutations have not been identified. Many proteins are involved in the process of EC coupling[3 9] and skeletal muscle calcium homeostasis. Consequently, there are a large number of potential candidate genes for MH susceptibility.

Polymerase chain reaction (PCR) has been used extensively to amplify specific regions of the human genome for subsequent DNA sequence analysis and has been successful in identifying novel mutations associated with MH.[4 10] This method, however, has limitations. Whereas amplifying individual genes by PCR to screen for disease-causing mutations in individuals is feasible, the time and cost associated with screening multiple candidate genes increase dramatically. With the advent of targeted genomic DNA capture, it is now possible to enrich human genomic DNA for specific target sequences[11] using microarray or in solution hybridization. Combining this method with next-generation sequencing not only increases the speed of DNA sequencing from genomic DNA samples but also lowers the cost per nucleotide. Therefore, it is now possible to analyse multiple candidate genes simultaneously to identify mutations causing disease.[12 13] The aim of this study was to investigate a new method, genomic DNA capture coupled with next-generation sequencing, to screen multiple candidate genes for their potential involvement in MH.

Methods

Patients

Patient A5 experienced an MH reaction during surgery for tonsillectomy, adenoidectomy, and insertion of ventilating tubes at 5 yr of age. There was no family history of anaesthetic problems. The mutational hotspot regions of *RYR1* (exon 6, 8–12, 14, 15, 17, 39–41, 43–47, 95, 98–104) had been previously screened for mutations using PCR and Sanger sequencing for one individual (A), although none had been found.

Patient B5 comes from a large discordant family; five members of this family suffered fatal MH reactions. A branch of the family (including patient B5) lacks the familial *RYR1* mutation (Thr4826Ile),[14] and in addition, MHS is not linked to chromosome 19.

Patients A and B5 were both diagnosed MHS by *in vitro* contracture testing (IVCT).

Blood and tissue samples

Blood and tissue samples were obtained with informed consent from the patients at the time of collection. Ethical approval was obtained from the Central Region (Wellington, New Zealand) and Massey University (Palmerston North, New Zealand) ethics committees.

In vitro contracture testing

Muscle biopsies were obtained and IVCT was performed at Palmerston North Hospital as part of the normal diagnostic procedures for MH-susceptibility according to the European Malignant Hyperthermia Group protocol[15] and our own control data.[16 17]

DNA and RNA extraction

Total RNA was isolated from 100 mg frozen skeletal muscle tissue using TRizol® Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Genomic DNA was isolated from leukocytes and lymphoblastoid cells using the Wizard™ DNA extraction kit according to the manufacturer's instructions (Promega, Madison, WI, USA).

Capture and sequencing of genomic DNA/data analysis

Genomic DNA (20 μg) was used for hybridization to custom-designed long-oligo NimbleGen arrays (385K). Sequencing (identifying the order of the DNA bases) was done using a system called shotgun sequencing. This is a method for determining the sequence of very large lengths of DNA. In shotgun sequencing the DNA is fragmented and the smaller pieces inserted into a convenient vector (a plasmid, usually) to replicate them, or amplified by PCR. The fragments are sequenced—i.e. the order of the nucleotides is determined and the sequence of the original large DNA sections is deduced from overlapping sections. The order of the bases is referred to as a 'read'. Sequencing was performed using GS FLX Titanium chemistry (Roche, Mannheim, Germany). Bioinformatic analysis of DNA variants was performed by Roche using GS FLX Data Analysis Software Suite V2.3, specifically the GS Reference Mapper application. All variants were screened against dbSNP build 131 (http://www.ncbi.nlm.nih.gov/snp/). [Single-nucleotide polymorphisms (SNPs) are DNA nucleotide variations in the genomes of individuals.] ClustalW2 was used for protein sequence alignments (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

High-resolution melting analysis

High-resolution melting (HRM) is a post-PCR analysis that examines the DNA melting curve and therefore can detect single basepair changes in the amplicon. HRM analysis was carried out as described previously.[18] Primers were designed using the LightCycler Probe Design Software 2.0 (Roche). Real-time PCR and HRM analysis of the genomic DNA samples were carried out on the LightCycler 480 System (Roche), using the SsoFast™ EvaGreen® Supermix (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. In brief, the HRM reaction mix contained 1× SsoFast™ EvaGreen® Supermix, 0.3 μM of each primer, and 10–70 ng of genomic DNA. Assays were performed in 96-well plates in a 10 μl volume using the following conditions: activation at 98°C for 2 min, followed by 45 cycles of 98°C for 5 s and 58°C for 5 s. After amplification, the samples were heated to 95°C for 1 min (25 acquisitions per 1°C) and then cooled to 40°C for 30 s. Information about the primers used for HRM analysis is available on request.

Reverse transcriptase–PCR and Sanger sequencing

The enzyme reverse transcriptase is used to transcribe single-stranded RNA into DNA, which is then amplified by

PCR. The DNA sequence of the amplified region is then determined by Sanger sequencing. Total RNA was first treated with TURBO DNase (Ambion, Carlsbad, CA, USA), then 1.5 μg RNA was used to synthesize first strand cDNA using Transcriptor First-Strand cDNA synthesis kit (Roche) according to the manufacturer's instructions. Dideoxysequencing was carried out using the BigDye™ Terminator Version 3.1 kit on an ABI 3730 (Applied Biosystems, Foster City, CA, USA) sequencer. Information about the primers used for sequencing is available on request.

Immortalized B lymphoblastoid cell lines

B lymphocytes were isolated from whole blood and transformed with Epstein–Barr virus as previously described.[19] The presence of the variants in the lymphoblastoid cells was confirmed by HRM analysis.

Functional calcium assays

Changes in the intracellular Ca^{2+} concentrations were measured using 1×10^6 cells ml^{-1} (final volume 2 ml) from MHS patients and MH normal (MHN)[15–17] controls using a spectrofluorimeter with a magnetic stirrer after addition of increasing concentrations of 4-chloro-*m*-cresol. These experiments are described in detail in the Supplementary material.

Statistical analysis

Statistical analysis was performed using the Student *t*-test for paired samples or analysis of variance when more than two groups were compared. Origin software (v. 8.5.1, Microcal Software, Northampton, MA, USA) was used for statistical analysis and for generation of dose–response curves. Results were calculated as mean values (SEM) of *n* results.

Results

We have evaluated the feasibility of identifying mutations causing MH using genomic DNA capture and next-generation sequencing of a limited set of genes. Custom-designed oligonucleotides were used to target 32 genes (coding regions and non-coding regions) of two individuals (Patients A and B5) using NimbleGen sequence capture arrays (385K) and then sequenced with Roche 454 Titanium shotgun sequencing. Sequencing data were processed through the GS FLX Data Analysis Software Suite v2.3. The candidate genes (Supplementary Table S1) were selected because of their potential role in EC coupling and/or skeletal muscle calcium homeostasis or the immune response. A total of ≈ 3.1 megabases of DNA were targeted.

The target base coverage was 95.8% and 95.9% for individuals A and B5, respectively. Approximately 10% of the reads obtained were outside the target regions. The average read length was 388 bp (A) and 380 bp (B5), and the average fold coverage was 64.2× (A) and 58.4× (B5). Exon 91 of the *RYR1* gene was only partially covered by sequence reads and was therefore subsequently sequenced by Sanger sequencing after PCR amplification from genomic DNA.

We identified 4406 (A) and 4475 (B5) sequence differences in the target regions and out of these, 73 (A) and 57 (B5) sequence differences were located within exons. We also examined introns, splice sites, 5′ near gene regions, 5′ UTRs, and 3′ UTRs where sequence data were available. Fifty-seven (A) and 47 (B5) sequence variants resulted in synonymous changes, and 16 (A) and 10 (B5) sequence variants resulted in amino acid changes. These variants were compared with dbSNP build131 (www.ncbi.nlm.nih.gov/snp) and common SNPs were disregarded. Two different variants in both (A) and (B5) were not found in dbSNP build131 and therefore were potential candidates for MH-susceptibility. The variants (two variants in *RYR1* for Patient A and one of each variant in *RYR1* and *TRDN* for Patient B5) were analysed by HRM to examine their segregation with disease in the family.

In one individual (A), we identified two sequence variants in the *RYR1* gene that lead to amino acid changes (Table 1). One of them, Arg1583Cys, had been reported previously,[4] while Val2102Leu was novel. Segregation of both variants in the family was tested by HRM analysis and both segregated with MHS status (Fig. 1). The variants were confirmed by Sanger sequencing in both directions in all family members where DNA was available. We also confirmed expression of the two variants in muscle tissue by sequencing cDNA prepared from RNA isolated from muscle tissue of Patient A. The two variants were absent in 113 MHN individuals screened by HRM and were also absent in 33 unrelated MHS individuals where MH-associated mutations had not been identified.

In Patient B5, we identified a sequence variant in *RYR1* that leads to an Ile3253Thr change and a sequence variant in *TRDN* leading to a Gln456Arg change. However, both variants did not segregate with disease in this family branch (see Supplementary material).

In total, we identified 19 known SNPs in the exons of the *RYR1* gene in the two patients (Table 2) and one new variant that did not segregate with disease in this family branch (Ile3253Thr).

Table 1 RYR1 variants identified in MH patient A. p.c.m., putative causative mutation. Nucleotide numbering reflects location on NCBI human chromosome 19 (NCBI36/hg18)

Exon	Nucleotide change	Amino acid change	Role of mutation	Reference
33	g.43665809C>T	Arg1583Cys	MHS p.c.m.	Broman and colleagues[4]
39	g.43676861G>C	Val2102Leu	MHS p.c.m.	This study

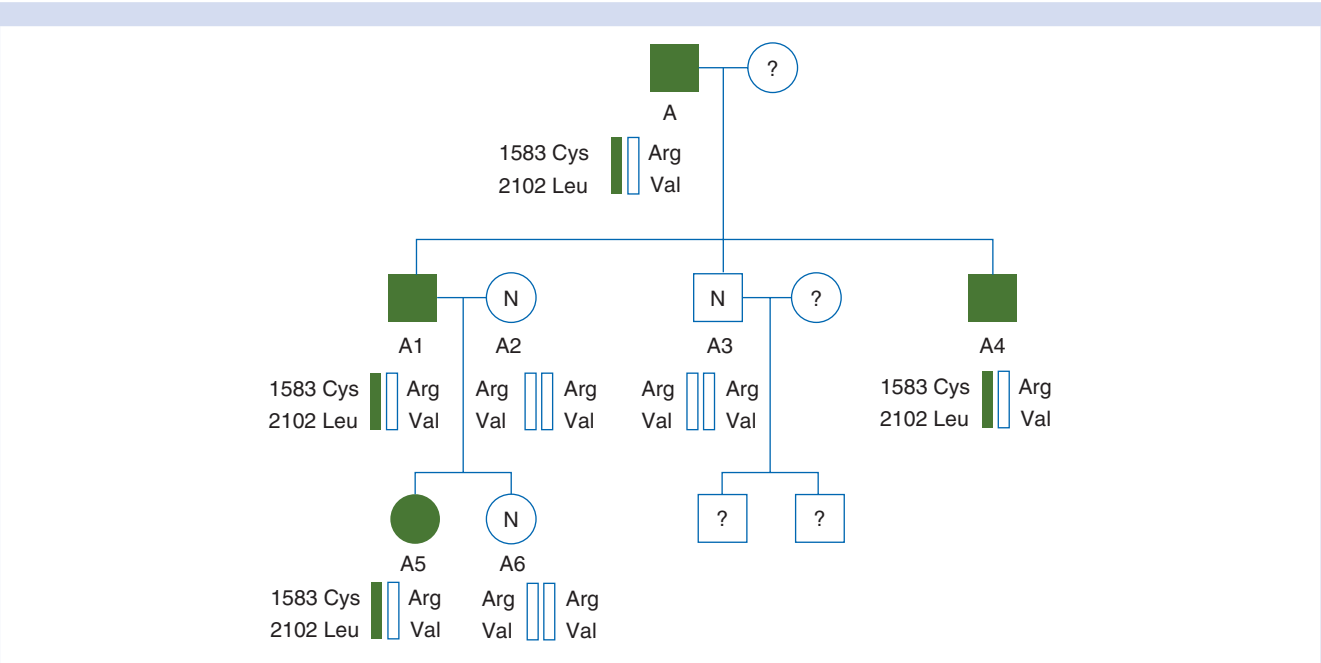

Fig 1 Segregation of the RYR1 variants Arg1583Cys and Val2102Leu. Filled symbols indicate MHS, open symbols with the letter N indicate MHN results, and open symbols with question marks indicate that IVCT has not been performed. A indicates the proband.

Table 2 SNPs detected in exonic regions of *RYR1* in both patients. Nucleotide numbering reflects location on NCBI human chromosome 19 (NCBI36/hg18).

Nucleotide change	Amino acid	Exon	dbSNP ID
g.43627120A>G	p.Leu 198	7	rs2229139
g.43631248T>C	p.Ala 359	11	rs10406027
g.43638022G>A	p.Ser 556	15	rs2288888
g.43641744C>T	p.Pro 762	19	rs3745847
g.43648643G>A	p.Thr 981	24	rs2228069
g.43648679C>T	p.Asn 993	24	rs2228070
g.43651520C>T	p.Ile 1152	26	rs11083462
g.43682296C>T	p.Arg 2403	44	rs78795178
g.43682433C>T	p.His 2420	45	rs12973632
g.43685396C>T	p.Arg 2624	49	rs1469698
g.43686750G>A	p.Thr 2659	50	rs2229144
g.43687278T>C	p.Ile 2706	51	rs2960340
g.43687350T>C	p.Asp 2730	51	rs2915951
g.43687815G>A	p.Glu 2779	53	rs2915952
g.43688830T>C	p.Ser 2863	55	rs2229146
g.43694565A>G	p.Pro 3062	62	rs2071089
g.43717206C>G	p.Gln3756Glu	79	rs4802584
g.43725891T>A	p.Thr 3918	85	rs45613041
g.43760481A>C	p.Thr 4752	98	rs1468571

The results of the functional Ca^{2+} assays in response to 4-chloro-*m*-cresol in lymphoblastoid cell lines are shown in the Supplementary material.

Discussion

New technologies enable us now to sequence the whole exome or specific genes of individuals. This is achieved by capturing the RNA coding regions in the genome by hybridizing genomic DNA to oligonucleotide probes. By selecting 32 genes, we increased the overall coverage of the targeted regions compared with coverage achieved when sequencing the whole exome. On the other hand, with whole exome sequencing, we would have included the vast majority of the expressed regions of the human genome.

In the current study, we sequenced 32 genes with a potential involvement in EC coupling, skeletal muscle calcium homeostasis, or immune response in two individuals with MH. Apart from *RYR1*, we also sequenced the two most relevant genes *CACNA1S* and *CASQ1*. So far, three MH-associated mutations have been reported in *CACNA1S*.[6 7 20] Mutations in *CASQ1* null mice cause an MH-like phenotype;[8 21] however to date, no mutation in *CASQ1* in humans has been associated with MH. We achieved 95% target coverage, which means that potential mutations in unrepresented gene regions would not have been detected. For example, exon 91 of the *RYR1* gene was only partially represented in both patient samples, probably because of its high GC content (the GC content of regions in the genome refers to the percentage of guanine and cytosine bases in the DNA; regions with a high GC content are more stable than regions with a low GC content). It has been reported previously that highly GC-rich regions have a lower capture efficiency.[12 22] We therefore used Sanger sequencing to cover the missing regions of this exon. To reduce the number of candidate

Table 3 IVCT data of family A. Maximal contractures in IVCT at the threshold concentrations of caffeine (2 mmol litre^{-1}) and halothane (2%)

Patient	Caffeine (g)	Halothane (g)	Diagnosis	Sequence variant or mutation found in *RYR1*
A	2.7	2.2	MHS	Arg1583Cys, Val2102Leu
A1	1.9	2	MHS	Arg1583Cys, Val2102Leu
A2	0.1	0.4	MHN	
A3	0.2	0.2	MHN	
A4	2.4	2.1	MHS	Arg1583Cys, Val2102Leu
A5	0.8	1.4	MHS	Arg1583Cys, Val2102Leu
A6	0	0.1	MHN	

mutations, we excluded common variants that were listed in dbSNP build131 (www.ncbi.nlm.nih.gov/snp/). We found that one causative mutation (www.emhg.org) in the ryanodine receptor (Gly248Arg) is listed in dbSNP build131 (rs1801086).

In one patient DNA sample, we detected an Arg1583Cys change in exon 33 and a Val2102Leu change in exon 39 of the *RYR1* gene which could be the potential cause of MH in this family as both changes segregate with MH in the family. Interestingly, Val 2102Leu was not detected in the mutational hot-spot screening. Other groups have also reported that they had not detected mutations using one method but were successful using an alternative method.[23] The mutation Arg1583Cys has been reported previously in connection with MH.[4] Interestingly, this group found that Arg1583Cys was linked to other variants in the *RYR1* gene, namely Gly248Arg (causative mutation) and Glu5034Val (polymorphism). Arg at position 1583 and Val at position 2102 are both conserved between most mammalian RYR1, but not between RYR2 and RYR3 isoforms.

B lymphocytes have been shown to express RYR1[24] and various studies have been published[10 25] that demonstrate that mutations in *RYR1* cause an increased sensitivity to agonists. The results of our functional assays (see Supplementary material) corresponded well with the IVCT data from the patients (Table 3). To determine if both candidate mutations can cause MH independently, further functional assays will need to be carried out using HEK293 cells transfected with mutated *RYR1* constructs.[26]

Although we screened 32 genes, we could not identify a likely mutation responsible for MH in the second patient. The *RYR1* Ile3253Thr change was not found in the father (B1, MHS) of B5, but was found in the mother (B2) who has no recorded family history of MH. We cannot completely rule out a possible role of the above variant in MH as we do not have IVCT results for B2. We believe the most likely scenario is that MH is caused by a mutation in a different gene involved in EC coupling or calcium homeostasis. Patient B5 had been previously screened for the R503C mutation in *CPT2* which has been associated with MH, but this mutation was absent. Another likely explanation could be that the mutation is located in one of the areas in the genome that was under-represented in this study. The family tree (see Supplementary material) suggests an autosomal-dominant inheritance, but we cannot rule out the possibility that there is more than one gene contributing to MH in this family. The influence of synonymous mutations on disease should also not be overlooked.[27]

Genomic capture and next-generation sequencing is becoming a relatively affordable and efficient method of screening candidate genes for their involvement in disease. This method has been successfully used to identify mutations in genes involved in breast and ovarian cancer and ataxia.[12 13] We successfully identified two putative mutations in the *RYR1* gene that could be the cause for MH in one family (A) and have shown that lymphoblastoid cells from affected patients are hypersensitive to 4-chloro-*m*-cresol. The search for a candidate gene causing MH in the other family (B) will need to be extended, possibly using whole exome sequencing. Nevertheless, we have clearly demonstrated the efficacy of next-generation sequencing and identified potential mutations associated with MH.

Supplementary material

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

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Declaration of interest

None declared.

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