Mutation screening of the RYR1 gene and identification of two novel mutations in Italian malignant hyperthermia families

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Abstract

Point mutations in the ryanodine receptor (RYR1) gene are associated with malignant hyperthermia, an autosomal dominant disorder triggered in susceptible people (MHS) by volatile anaesthetics and depolarising skeletal muscle relaxants. To date, 17 missense point mutations have been identified in the human RYR1 gene by screening of the cDNA obtained from muscle biopsies. Here we report single strand conformation polymorphism (SSCP) screening for nine of the most frequent RYR1 mutations using genomic DNA isolated from MHS patients. In addition, the Arg163Cys mutation was analysed by restriction enzyme digestion. We analysed 57 unrelated patients and detected seven of the known RYR1 point mutations. Furthermore, we found a new mutation, Arg2454His, segregating with the MHS phenotype in a large pedigree and a novel amino acid substitution at position 2436 in another patient, indicating a 15.8% frequency of these mutations in Italian patients. A new polymorphic site in intron 16 that causes the substitution of a G at position -7 with a C residue was identified.

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Malignant hyperthermia (MH) is a heterogeneous genetic disorder characterised by autosomal dominant transmission with incomplete penetrance and variable expressivity. MH is triggered in susceptible subjects by inhalational anaesthetics and depolarising muscle relaxants.[1] The diagnosis of MH susceptibility is achieved by the in vitro contracture test (IVCT) in which strips of biopsied skeletal muscle are exposed to caffeine and halothane. Levels of tension developed in response to the addition of caffeine at a concentration of 2 mmol/l or less and of halothane at a concentration of 2% v/v or less allow different diagnoses to be made: MH susceptible (MHS), where the muscle biopsy responds with an increase of 0.2 g in muscle tension; MH normal (MHN), where the threshold tension is not reached for caffeine or halothane; MH equivocal (MHE), where the threshold tension is reached for one agent only.

A similar syndrome is also present in pigs, where it has been shown that the primary defect resides in a single point mutation (Arg614Cys) in the porcine RYR1 protein.[2] The human RYR1 gene is localised on chromosome 19 at q12-13.2 and is composed of 106 exons spanning a genomic region of 160 kb.[3 4] Linkage analysis and mutation screening showed that mutations in the RYR1 gene are responsible for about 30-40% of human MHS patients. To date, several point mutations have been described in the human RYR1 gene: Cys35Arg,[5] Arg163Cys,[6] Gly248Arg,[7] Gly341Arg,[8] Tyr522Ser,[9] Arg552Trp,[10] Arg614Cys,[11] Arg614Leu,[12] Gly2435Arg,[13 14] Arg2436His,[15] Arg2163Cys, Arg2163His, Val2168Met, Thr2206Met,[16] Arg2458Cys, and Arg2458His.[17] Among these point mutations, Arg163Cys, Tyr522Ser, and Arg2436His have been identified in MHS families who also have central core disease (CCD), a rare myopathy characterised by hypotonia and proximal muscle weakness, closely associated with MH.[18] One additional mutation, Ile403Met, was found only in association with CCD.[6] The known mutations are localised at the 5' end (exons 2, 6, 9, 11, 12, 14, 15, 17) and in the middle (exons 39, 45, 46) of the gene. Fifteen of these point mutations have been incorporated into the RYR1 gene and have been shown to alter the activity of the RYR1 protein.[19]

Linkage analysis based on IVCT phenotyping showed that RYR1 mutations are not responsible for all the MH families,[20] indicating genetic heterogeneity in MH susceptibility. In agreement, a second MH locus on chromosome 1q was identified and a point mutation in the CACNL1A3 gene coding for the α1 subunit of the dihydropyridin receptor (DHPR) was found to segregate with the MH phenotype in one pedigree.[21] Clear linkage between MH and markers defining a 1 cM interval on chromosome 3q13.1 has been described.[22 23] A fourth locus on chromosome 7q21-22, where the gene encoding the α2/δ subunit of the DHPR maps, has been linked to MH in a single family, but no causal mutations have been discovered yet.[24]

Since the known mutations are clustered at the 5' end and in the middle of the RYR1 gene, we set up single strand conformation polymorphism (SSCP) screening for the most frequent mutations using genomic DNA isolated from MHS patients. In particular, we designed PCR primers on the intronic sequences flanking the exons and set up SSCP conditions for the

Table 1 Primers and PCR conditions for the analysis of RYR1 exons

Exon	*5' upper primer 3'* *5' lower primer 3'*	*Size (bp)*	*T_{ann} (°C)*	*Mg^{2+} (mmol/l)*
11	GCTCCCCTGCTAAACACACAG CCTGGACTCAAGCGATTCTCC	279	63	1.2
17	CCCTCCCTGGGTTCTTCTGTAGAT TCTGGGTGTGGAGTCTCTAAGTCA	282	55	1.5
39	TGGTGCTCCAAGCCTTGCATTGTC CAAAGTAAGGGGAAGGGCGGTGTC	336	63	1.5
45	TGCCTCCCCAACCCACCCACCTTC TGCCTGCCCTCCCTTCCCATCCTG	183	63	1.5
46	TGCCCCTCTCCCTCCCTCTACTCC AGCATCACTCCTTCGCCAAGTTCC	185	63	1.5

analysis of five exons, in which nine frequently detected mutations occur, together with a restriction enzyme analysis for the detection of the Arg163Cys mutation. Using this approach, 57 unrelated MHS patients were analysed and nine RYR1 point mutations were detected. In particular we found a new mutation, G7361A (Arg2454His), segregating with the MH phenotype in a large pedigree and a novel amino acid substitution at position 2436 (Arg2436Leu). In addition we identified a new polymorphic site in intron 16 which causes the substitution of a G at position -7 for a C residue.

Methods

Fifty-seven unrelated MHS patients diagnosed according to the European protocol for the IVCT test were included in this study. Genomic DNA was prepared from blood samples. For the SSCP analysis we designed PCR primers on the intronic sequences[4] flanking RYR1 exons 11, 17, 39, 45, and 46 looking for mutations both in the coding region and in the splicing consensus sequence. We set up PCR and SSCP conditions for the detection of the more frequent nine point mutations (Gly341Arg, Arg614Cys, Arg614Leu, Arg2163Cys, Arg2163His, Gly2435Arg, Arg2436His, Arg2458Cys, and Arg2458His). The mutation Arg163Cys in exon 6 was analysed by specific restriction enzyme digestion.[6] These mutations account for about 20-25% of MHS cases.

Primer sequences and PCR conditions used for the SSCP analysis are reported in table 1 together with the expected size of the amplified fragments. PCR reactions were set up in a total volume of 50 µl using 200-300 ng of genomic DNA with 25 pmol of the corresponding forward and reverse primers in 10 mmol/l Tris-HCl, 50 mmol/l KCl, 1.2-1.5 mmol/l $MgCl_2$ (pH 8.3) amplification buffer containing 1.25 U *Taq* polymerase and 200 µmol/l of each dNTP. Thirty-five cycles of amplification were performed in each case at 94°C for 40 seconds, at the annealing temperature (table 1) for 30 seconds, and at 72°C for 30 seconds. The first denaturation step was performed at 94°C for five minutes and the last elongation was at 72°C for five minutes. Different gel compositions were used for each exon in order to increase the probability of detecting SSCP variant bands.[25 26] In particular 4-8 µl of PCR product were loaded on a non-denaturing polyacrylamide gel containing 6% acrylamide prepared with a 99:1 ratio between acrylamide and bis-acrylamide and alternatively without glycerol or with 5% glycerol in either 1 × or 0.5 × TBE. Gels were run in a cold room for one to four hours at 35 W. DNA bands on the gel were visualised by silver staining according to a protocol previously described.[27] After SSCP analysis, every sample showing a conformational variant was sequenced using the Sequenase 2.0 kit (USB) directly from the purified PCR product. Since the PCR product from exon 39 has a size of 336 bp, two different conditions were applied for this exon in order to increase the probability of detecting SSCP conformational variants. In particular, the PCR product was run as full length and after digestion with *Bst*UI restriction enzyme which releases two fragments of 175 and 161 bp.

The polymorphism in intron 16 ($G^{-7}\rightarrow C^{-7}$) causes the loss of a *Sac*I restriction site, resulting in two alleles (allele 1: 279 bp and 45 bp; allele 2: 282 bp). The analysis of the allele frequencies was therefore performed by digesting 10 µl of the PCR product obtained with exon 17 primers with 5 U of *Sac*I and running the digested fragments in a non-denaturing 8% polyacrylamide gel. The restriction fragments were visualised by ethidium bromide staining.

Results

Starting from the genomic organisation of the human RYR1 gene,[4] we designed primers in the intronic sequences flanking exons 11, 17, 39, 45, and 46 able to amplify the coding region and the splicing consensus sequence of these exons. This approach should allow the detection of all the nine known mutations and of any new ones occurring in this region. Accordingly, we detected five of the known mutations (Gly341Arg, Arg614Cys, Arg614Leu, Gly2435Arg, and Arg2458Cys). The remaining four (Arg2163Cys, Arg2163His, Arg2436His, and Arg2458His) were not observed in our patients. In addition, two new mutations in exons 45 and 46 were detected. The size of the amplified fragments for exons 11, 17, 45, and 46 was in the range between 183 and 282 bp, while exon 39 was larger than 300 bp. The exon 39 sequence contains a single *Bst*UI restriction site allowing us to cut it into two shorter fragments so as to improve the SSCP sensitivity. As already reported,[6] the Arg163Cys mutation causes loss of a *Bst*UI site and was therefore analysed by restriction enzyme digestion.

Table 2 summarises the point mutations that we found and the IVCT results. We found a mutation Arg163Cys in exon 6 in one MHS-CCD patient; a mutation in exon 11 (Gly341Arg) in one MHS patient and his affected mother; in exon 17 we found one MHS patient carrying the Arg614Cys mutation, who showed a minicore feature, and two patients carrying the Arg614Leu mutation; in exon 45 we found a mutation (Gly2435Arg) in one patient and her mother, a silent mutation in another patient (C7260T), and one G7304T transversion which results in a novel amino acid substitution at position 2436 (Arg2436Leu) in one MHS-CCD patient. A conformational variant in exon 46 in one

Table 2 IVCT results and RYR1 mutations found in nine MH families. The values given for each person are the average of the IVCT response of two muscle strips, except for subjects F17/14, F70/32, F93/86, F73/34, and F73/42 where data from three muscle strips were available. The new mutations are in bold. The vertical line indicates subjects from the same family (fig 1)

	Mutation	*Diagnosis*	*Contracture at 2% halothane (g)*	*Halothane threshold (%)*	*Contracture at 2 mmol/l caffeine (g)*	*Caffeine threshold (mmol/l)*
F79/61	Arg163CYS	MHS-CCD	6.02	0.5	3.99	0.5
F17/14	Gly341Arg	MHS	0.77	0.5	1.55	0.5
F70/32	Arg614Leu	MHS	1.04	0.5	2.45	1
F83172	Arg614Leu	MHS	0.63	0.5	0.9	1.5
F90/80	Arg614Cys	MHS-minicore	1.11	0.5	0.36	1.5
F3/2	Gly2435Arg	MFIS	0.45	0.5	0.5	1.5
F93/86	**Arg2436Leu**	MHS-CCD	6.22	0.5	5.97	0.5
|F73/34	**Arg2454His**	MHS	1.3	0.5	1.5	1
|F73/38	**Arg2454His**	MHS	1.15	0.5	1.05	1.5
|F73/42	**Arg2454His**	MHS	1.2	0.5	1.05	1.0
F88/77	Arg2458Cys	MHS-core	1.64	0.5	0.23	1.5

patient was found to correspond to the Arg2458Cys substitution. Histological examination of muscle biopsy tissue from a patient carrying the Arg2458Cys substitution showed a multicore feature with amorphous areas distributed in type 1 fibres. Another variant in exon 46 was found to give rise to a new mutation (G7361A) which causes the substitution of arginine at position 2454 with a histidine. Analysis of the DNA sequence surrounding the nucleotide change G7361A indicated that this mutation destroyed a *Bst*UI restriction site allowing us to assess the cosegregation of the mutated allele with the MH phenotype in a large pedigree (fig 1). Both the new mutations (Arg2454His and Arg2436Leu) were absent in 96 normal chromosomes analysed. Taken together, all the mutations found (9/57) account for a frequency of 15.8% in our group of MHS patients.

The analysis of exon 17 also led to the identification of a new polymorphic site $G^{-7} \rightarrow C^{-7}$ in intron 16. The allele frequency was estimated in 50 normal chromosomes and was 0.92 for allele 1 and 0.08 for allele 2. Since the alleles of this polymorphism were found in MHS patients at the same frequency as in the normal population, it probably has no significance with respect to the disease. Despite its low information content this polymorphism may be useful for population genetic studies.

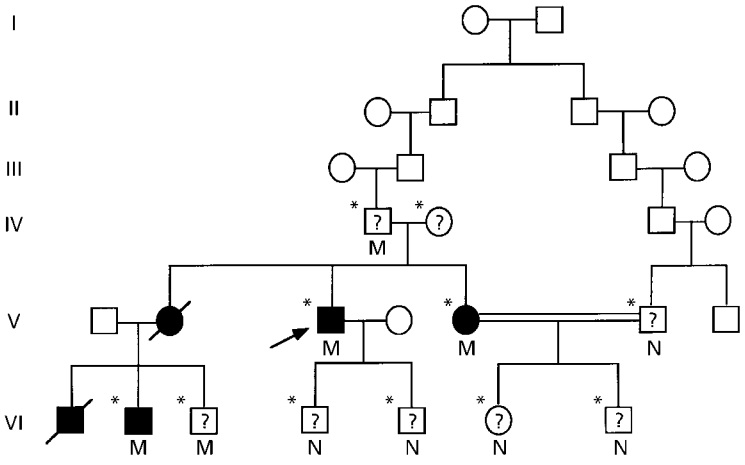

*Figure 1 Segregation analysis of the Arg2454His mutation. The proband is indicated by an arrow. V.2 and VI.1 died of a MH crisis. Filled symbols represent MHS subjects diagnosed by IVCT. ?: status unknown. M: mutation Arg2454His. N: mutation Arg2454His was not found. *DNA available for analysis.*

Discussion

Here we report the detection of the most frequent RYR1 mutations using genomic DNA. Since the mutations described so far are clustered at the 5' end and in the middle of the RYR1 gene,[16] it was possible to set up PCR-SSCP conditions for the analysis of five exons and their flanking consensus splicing sites. Using this approach we found nine point mutations in 57 Italian patients analysed for the presence of mutations in five RYR1 exons and for the presence of the Arg163Cys substitution, which corresponds to a frequency of about 15.8% of these RYR1 point mutations in Italian MHS patients. High IVCT contracture values were found in patients F79/61 and F93/86 carrying the Arg163Cys and the Arg2436Leu mutations respectively. However, a statistical analysis to evaluate the relationship between the IVCT phenotype and the amino acid substitution could not be done owing to the small number of patients carrying the same mutation. The identification of two novel potential mutations with the SSCP technique instead of restriction analysis screening of the known mutations illustrates one of the limitations in the use of restriction enzyme digestion for mutation detection in MH.

Recently, the Arg2163Cys substitution in exon 39 was reported with a frequency of 4%,[16] together with Arg2163His in a single family. Since we did not observe any mutation in exon 39, we were concerned that it could be because of the low efficiency of the SSCP technique in showing conformational variants of large sized PCR products. In order to increase the sensitivity of the SSCP technique, the exon 39 PCR product was digested into two smaller fragments, but still no conformational variants were observed, confirming that the Arg2163Cys and the Arg2163His mutations do not occur in our patients.

The two new mutations presented here satisfy the criteria for potential causative MHS mutations: neither Arg2454His nor Arg2436Leu were found in 96 normal chromosomes analysed and these amino acids are conserved across species and related isoforms indicating their functional significance. Furthermore, the Arg2454His mutation segregates with the MHS phenotype in the pedigree investigated here (fig 1, V.3, V.5, and VI.2),

allowing us to perform a presymptomatic diagnosis of MHS for the people (IV.1, VI.3, VI.4, VI.5, VI.6, and VI.7) not yet investigated by IVCT.

The relationship between MH and CCD is still unclear. In agreement with previous observations,[6] the Arg163Cys mutation was found in a MHS patient who also showed a feature of CCD. The Arg2436His mutation was reported[15] in a single, large, Canadian pedigree with recurrence of CCD. Interestingly, it has been shown that all CCD patients belonging to this family who have been tested have proven to be MHS, associating both MH and CCD with the same altered Ca^{2+} release channel. Here we report the discovery of a new RYR1 point mutation occurring at the same position 2436, but causing the substitution of the arginine with a leucine residue in a MHS patient with CCD. The same phenotypic expression of these two mutations suggests that the nature of the amino acid substitution (His and Leu) is not relevant with respect to the position of the mutation in the protein. Furthermore, the discovery of another mutation affecting Arg2436 supports the association between MH and CCD as a result of specific amino acid substitutions in the RYR1 protein.

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