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Detection of a novel mutation at amino acid position 614 in the ryanodine receptor in malignant hyperthermia

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Summary

Malignant hyperthermia (MH) is a potentially fatal autosomal dominant disorder of skeletal muscle and is triggered in susceptible people by all commonly used inhalation anaesthetics and depolarizing neuromuscular blocking agents. To date, eight mutations in the skeletal muscle ryanodine receptor gene (RYR1) have been identified in malignant hyperthermia susceptible (MHS) and central core disease (CCD) cases. We have screened the RYR1 gene in affected individuals for novel MHS mutations by single stranded conformational polymorphism (SSCP) analysis and have identified a G to T transition mutation which results in the replacement of a conserved arginine (Arg) at position 614 with a leucine (Leu). The Arg614Leu mutation was present in three unrelated MHS individuals of 151 investigated. The mutation was not detected in 148 normal chromosomes and segregated precisely with MHS in family members from one of the probands where DNA was available for analysis. This mutation occurs at the same position as the previously identified Arg to Cys mutation reported in all cases of porcine MH and in approximately 5% of human MH. A comparison of the phenotypes of the Arg614Leu and Arg614Cys probands is presented. (*Br. J. Anaesth*. 1997; **79**: 332–337).

Key words

Malignant hyperthermia. Genetic factors, hyperthermia. Receptors, ryanodine.

Malignant hyperthermia (MH) is a clinically heterogeneous autosomal dominant disorder elicited in susceptible individuals by widely used volatile anaesthetics and depolarizing neuromuscular blocking agents and characterised by skeletal muscle hypermetabolism.[1 2] The reported incidence of MH ranges from approximately 1 per 10 000 to 1 per 50 000 anaesthetics with an apparently higher incidence in children.[3]

MH also occurs in pigs and is closely associated with muscle hypertrophy. Biochemical studies on porcine MH indicate that the primary defect in MH susceptible (MHS) cases is in the skeletal muscle calcium release channel, commonly termed the ryanodine receptor (RYR1).[4] Sequence analysis of the porcine RYR1 gene has shown that a point mutation is present in MH pigs that results in an arginine (Arg) to cysteine (Cys) substitution at position 615 (Arg615Cys).[5] A mutation equivalent to the pig Arg615Cys has also been found in human MH.[6] The human mutation is known as the Arg614Cys mutation and results from a C1840T transition mutation.

In addition to this mutation, another seven RYR1 point mutations have been identified in MHS and central core disease (CCD)[7] pedigrees, namely Arg163Cys,[8] Gly248Arg,[9] Gly341Arg,[10] Ile403Met,[8] Tyr522Ser,[11] Gly2433Arg[12 13] and Arg2434His.[14] Collectively, these mutations have been found in approximately 10% of Caucasian MHS cases. Identification of these mutations firmly establishes the RYR1 gene as a susceptibility gene in MH and indicates that mutations are likely to be present in the RYR1 gene in all RYR1 linked families.

In an effort to identify new mutations in the RYR1 gene causing MH we have undertaken systematic screening of the 15 099 bp coding sequence of the RYR1 gene in unrelated MHS individuals for mutations. We describe the identification of a novel Arg614Leu mutation which we detected in a MHS pedigree and two unrelated MH probands.

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Patients and methods

Screening for single nucleotide changes in the RYR1 gene of MHS patients requires amplification of the entire gene from each individual in short segments and subsequent investigation of each of these gene segments for nucleotide changes. The RYR1 gene spans 158 kb of genomic DNA and contains more than 100 exons.[15] For amplification purposes, we chose to use RYR1 complementary DNA (cDNA) as muscle biopsy material was available from MHS individuals. For synthesis of cDNA, total RNA was first extracted by guanidinium thiocyanate[16] from the muscle tissue and first strand cDNA synthesis was performed as described previously.[10] PCR primers were designed using the computer programme OLIGO version 4.0,[17] so that the entire 15 099 bp coding sequence of the RYR1 gene[18] could be amplified in short (200–450 bp) overlapping segments, using cDNA as template in the amplification reaction (the sequence of the 64 PCR primer pairs and PCR conditions are available on request).

Screening for single nucleotide changes in RYR1 segments amplified by PCR from the MHS patients was performed using the well established single stranded conformation polymorphism (SSCP) method[19] under three different electrophoretic conditions, as described previously.[10] The DNA bands on the polyacrylamide gel were detected by autoradiography. Normal samples were included in the SSCP analysis as controls to allow identification of normal alleles.

Sequence analysis of PCR amplified fragments exhibiting aberrant SSCP patterns was performed as described previously.[10]

For segregation studies, genomic DNA was extracted from peripheral blood as described previously.[20] A 74-bp fragment spanning the G1841T mutation of the RYR1 gene was amplified from genomic DNA using PCR, as described previously.[6] For SSCP detection, the samples were run on a 6% polyacrylamide gel (acrylamide N, N'methylenebisacrylamide = 99:1) with 5% glycerol at room temperature for 1.5 h. The G1841T SSCP was detected by autoradiography.

For the investigations reported here, muscle samples were obtained from 29 MHS patients and five MH negative (MHN) individuals through the Lille, Copenhagen and Ulm MH testing centres. Patients were tested for MH susceptibility using the standardized European Malignant Hyperthermia Group IVCT procedure.[21]

Results

SSCP ANALYSIS OF THE RYR1 GENE IN MH

SSCP polyacrylamide gel band patterns, which did not correspond to those observed in normal individuals, were detected in eight areas of the RYR1 gene in patient 12F. Seven of these were considered to be polymorphisms (normal variations in the DNA sequence which do not result in an abnormal protein product[9 14]) as they were present in some of the control samples (normal individuals) and/or were found to be silent mutations after DNA sequence analysis. However, direct sequencing of the RYR1 segment 1727–1993 in patient 12F, which also demonstrated an aberrant SSCP pattern, revealed the presence of the single base substitution G1841T which results in the replacement of an Arg with a Leu at position 614 (fig. 1).

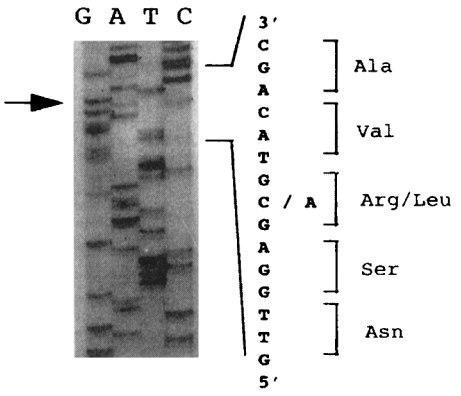

Figure 1 Nucleotide sequence of the mutated region in RYR1 1727–1993 segment from patient 12F. Sequencing was carried out using alpha-32P dCTP and was performed with the reverse primer 1993 so that the sequence of the reverse strand was obtained. The position of the C1841A transition on the reverse strand sequence (G1841T on the forward strand) is indicated by the arrow.

The presence of the G1841T missense mutation in genomic DNA extracted from blood samples was verified by checking the appropriate amplified products for the presence of the SSCP banding pattern characteristic of the presence of the mutation and by direct sequencing.

To establish if the Arg614Leu mutation was present in the normal population, 148 normal chromosomes were examined for the presence of the mutation. None displayed the candidate mutation. In order to determine if these mutations were present in the RYR1 gene of other MHS pedigrees or CCD pedigrees, 122 additional

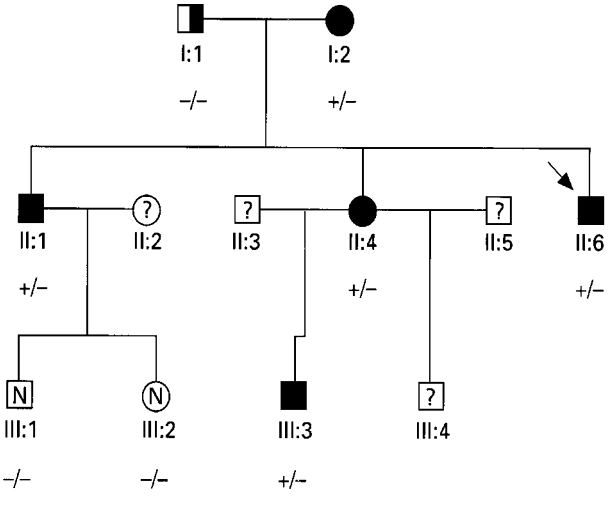

Figure 2 Pedigree of MH proband D15. Symbols: black = MHS; open = MHN; half-black = MHE(h); open with question mark = disease status unknown. The proband (individual II-6) is indicated by an arrow. Segregation of the Arg614Leu RYR1 mutation is also indicated: + = presence of the mutant allele; – = presence of the normal allele.

Table 1 Results of the *in vitro* contracture test in (A) individuals with the Arg614Leu mutation, including members of pedigree D15 and (B) probands with the Arg614Cys mutation. The halothane and caffeine thresholds are the concentrations of halothane (%) and caffeine (mmol litre^{-1}), respectively, at which a contracture of 0.2 g tension is generated. *Taken from.[22] NA = not available.

	Family	ID	Diagnosis	Halothane threshold (%)	Contracture at 2% halothane (g)	Caffeine threshold (mmol litre^{-1})	Contracture at 2 mmol litre^{-1} caffeine (g)	RYR1 mutation
A	D15	I-1	MHE(h)	2.0	0.4	3.0	0	Arg614Leu absent
		I-2	MHS	0.5	3.2	0.5	2.1	Arg614Leu present
		II-1	MHS	0.5	2.5	0.5	1.85	Arg614Leu present
		II-4	MHS	0.5	4.15	1.0	2.3	Arg614Leu present
		II-6 (Proband)	MHS	0.5	1.8	1.0	1.0	Arg614Leu present
		III-1	MHN	>3.0	0	4.0	0	Arg614Leu absent
		III-2	MHN	>3.0	0	4.0	0	Arg614Leu absent
		III-3	MHS	0.5	1.4	0.5	1.7	Arg614Leu present
	1GR	Proband	MHS	0.5	Fibres disrupted	0.5	1.0	Arg614Leu present
	12F	Proband	MHS	0.5	1.5	1.5	0.58	Arg614Leu present
B	G2	Proband	MHS	1.0	0.2	1.5	0.8	Arg614Cys present
	G3	Proband	MHS	2.0	0.5	2.0	0.2	Arg614Cys present
	G7	Proband	MHS	0.5	Fibres disrupted	0.5	0.64	Arg614Cys present
	10F	Proband	MHS	NA	0.9	NA	0.23	Arg614Cys present
	8F	Proband	MHS	0.5	NA	NA	0.25	Arg614Cys present
	1F	Proband	MHS	2.0	0.2	1.0	0.3	Arg614Cys present
	MH011*	302	MHS	1.0	ND	2.0	0.2	Arg614Cys present
	MH011*	408	MHS	0.5	0.5	0.5	ND	Arg614Cys present
	MH011*	503	MHS	1.0	0.9	2.0	0.8	Arg614Cys present
	MH011*	501	MHS	0.5	2.5	1.5	1.3	Arg614Cys present
	MH011*	506	MHN	>3.0	0.0	>4.0	0.0	Arg614Cys present

Table 2 Clinical indicators (abnormal signs and laboratory findings) recorded during MH reactions and used to determine the MH raw score in the case of three unrelated Arg614Leu probands (R614L) and nine unrelated Arg614Cys probands (R614C).[23] ND = not determined

Individual	Anaesthetic administered	Suxamethonium administered	Process I: Rigidity	Process II: Muscle breakdown	Process III: Respiratory acidosis	Process IV: Temperature increase	Process V: Cardiac involvement	Other indicators	Raw score
GR1 (R614L)	Halothane	Yes	0	15	0	0	3	ND	18
12F (R614L)	Thiopentone Isoflurane	No	0	15	15	15	3	5	53
D15 (R614L)	Thiopentone Halothane	Yes	15	10	10	15	3	ND	53
G2 (R614C)	Halothane	Yes	15	5	0	0	3	5	28
G3 (R614C)	Isoflurane	Yes	15	15	15	15	3	25	88
G4 (R614C)	Halothane	Yes	15	15	ND	15	3	5	53
G5 (R614C)	Halothane	Yes	15	15	ND	0	ND	ND	30
G6 (R614C)	Enflurane	Yes	ND	ND	15	10	3	25	53
G7 (R614C)	Halothane	Yes	0	ND	ND	10	0	25	35
1F (R614C)	Halothane	No	15	15	ND	0	3	ND	33
8F (R614C)	Halothane	No	15	15	15	15	3	5	68
10F (R614C)	Halothane	No	ND	ND	ND	15	3	ND	18

unrelated MHS and five unrelated CCD patients were investigated for the presence of these mutations. The mutation was detected in one Danish (D15) and one German (GR1) MHS patient.

The family of individual D15 (fig. 2) was investigated to determine if the Arg614Leu mutation co-segregated with the MHS phenotype. The mutation co-segregated with the MHS phenotype in this family (fig. 2). The families of individuals 12F and GR1 were unavailable for analysis.

Interestingly, Arg614 is the position of the original porcine RYR1 mutation (Arg615Cys and Arg614Cys in pigs and humans, respectively) which accounts for all cases of porcine MH investigated to date and for approximately 5% of human MH cases.[6] As Arg614 is the position of two MH causative mutations, it is of interest to compare the contracture data and clinical phenotypes. Table 1 presents the IVCT results of individuals carrying the Arg614Leu mutation (pedigree of D15, 1GR, 12F) and results of individuals carrying the Arg614Cys mutation, including six MH probands and five individuals from pedigree MH011 described by Deufel and colleagues.[22] Individual 408 of the MH011 pedigree is homozygous for the Arg614Cys mutation although the two C1840T base transitions segregate independently. Individual 506 who is heterozygous for the Arg614Cys mutation was diagnosed MHN by the IVCT and is the only reported MHN individual carrying the Arg614Cys mutation.[22]

The contracture thresholds (the concentration of caffeine or halothane at which a contracture of 0.2 g tension is generated) of the three probands with the Arg614Leu mutation varied significantly for caffeine (0.5–1.5 mmol litre^{-1}) whereas the halothane threshold was the same in all three cases (0.5%)

(table 1). Individual 506 of pedigree MH011, who is MHN and heterozygous for the Arg614Cys mutation, registered a caffeine threshold >4.0 mmol litre^{-1} and a halothane threshold $>3.0\%$. The contracture thresholds of MHS individuals with the Arg614Cys mutation ranged from 0.5 to 2.0 mmol litre^{-1} for caffeine and from $<0.5\%$ to 2.0% for halothane.

In an effort to compare the clinical phenotype of MHS individuals with the Arg614Cys and Arg614Leu mutations, we applied the MH clinical grading scale devised by Larach and colleagues[23] (table 2). In this system, the quantitative likelihood that an adverse anaesthetic event represents MH is based on points assigned to specific abnormal signs and laboratory findings (clinical indicators) observed during an acute anaesthetic reaction. Points are assigned for each indicator present. As several agreed upon indicators are the manifestations of the same process, only the single indicator with the highest score within a given process would count towards the raw score. These points are then summed to produce a raw score that an adverse anaesthetic reaction is a MH event.

Discussion

In an ongoing effort to identify new mutations in the RYR1 gene causing MHS, we have investigated the RYR1 gene in unrelated MHS patients for the presence of new mutations. Analysis of the RYR1 cDNA sequence was performed in preference to analysis of the genomic DNA sequence (>100 exons) as only 64 PCR primer pairs are required for the former while more than 100 primer pairs are required for the later.[15] Furthermore, as muscle biopsy samples from MHS individuals are readily available after the IVCT, availability of RNA for cDNA synthesis is not a limiting factor. The RYR1 PCR primers described here work reproducibly to give good quality PCR products suitable for SSCP analysis and DNA sequencing.

In this study, we detected a novel Arg614Leu mutation in three of 151 unrelated Caucasian MHS probands. The pedigree of one of these probands was available for analysis and the Arg614Leu mutation segregated with MHS in this pedigree (fig. 2). Individual I-1 of this pedigree, diagnosed as MHE(h) by the IVCT, was negative for the presence of the mutation. Examination of the contracture records showed that this individual exhibited a significant response to halothane (0.4 g at 2% halothane). The observation that some patients can produce significant responses in the IVCT in the absence of the reported segregating mutation has been reported previously by us in families where the Gly341Arg and Gly2433Arg mutations segregated with MHS.[10 12]

The Arg614Leu mutation was not detected in 148 normal chromosomes. Furthermore, examination of the RYR1 genes sequenced to date[5 17] and related isoforms[24–27] indicate that Arg614 is a functionally important amino acid in the RYR proteins as it is conserved across species (fig. 3). Therefore, the Arg614Leu mutation is not likely to be a coincidental polymorphism and is likely to cause MHS in patients bearing the mutation.

The Arg614Cys and Arg614Leu mutations are the result of a C1840T and G1841T transition, respectively, at a CpG dinucleotide in the RYR1 gene sequence. Methylation of a CpG dinucleotide to 5-methyl cytosine creates a mutation hot spot because of the high frequency of deamination of 5-methyl cytosine to thymidine, resulting in the TpG dinucleotide and the complementary dinucleotide CpA.[28] CpG dinucleotides are the site of approximately 35% of all known disease causing point mutations and of these, more than 90% are accounted for by C to T or G to A transitions.[29] In contrast, the Arg614Leu mutation is the result of a G1841T transition at this CpG dinucleotide.

As Arg614 is the position of two mutations potentially causative of MH, we compared the contracture data and clinical phenotypes of probands (tables 1 and 2). Comparison of the caffeine and halothane contracture thresholds (the concentrations at which a contracture of 0.2 g tension is generated) of individuals carrying the Arg614Leu and Arg614Cys mutations (table 1) suggests that the Arg614Leu mutation exhibits a more severe phenotype in the IVCT.

Mutations in the RYR1 gene that cause MH are thought to make the Ca^{2+} release channel sensitive to lower concentrations of agonists.[30] This hypersensitive gating of the channel enhances rates of Ca^{2+} release from the sarcoplasmic reticulum during anaesthesia which results in sustained muscle contraction and sustained glycolytic and aerobic metabolism associated with MH.[30] The suggestion of a more potent effect of the Arg614Leu mutation compared with the Arg614Cys mutation in the IVCT could be explained by increased sensitivity of the RYR1 protein with the Arg614Leu mutation to

							614								
Human skeletal	Val	Cys	Asn	Gly	Val	Ala	Val	Arg	Ser	Asn	Gln	Asp	Leu	Ile	Thr	Glu
Pig skeletal	Val	Cys	Asn	Gly	Val	Ala	Val	Arg	Ser	Asn	Gln	Asp	Leu	Ile	Thr	Glu
Rabbit skeletal	Val	Cys	Asn	Gly	Val	Ala	Val	Arg	Ser	Asn	Gln	Asp	Leu	Ile	Thr	Glu
Rabbit cardiac	Val	Cys	His	Gly	Val	Ala	Val	Arg	Ser	Asn	Gln	His	Leu	Ile	Cys	Asp
Rabbit brain	Leu	Cys	Asn	Gly	Val	Ala	Val	Arg	Ala	Asn	Gln	Asn	Leu	Ile	Cys	Asp
Bullfrog alpha	Val	Cys	Asn	Gly	Val	Ala	Val	Arg	Ser	Asn	Gln	Asn	Leu	Ile	Thr	Glu
Bullfrog beta	Val	Cys	Asn	Gly	Val	Ala	Val	Arg	Thr	Asn	Gln	Asn	Leu	Ile	Cys	Asp
Drosophila	Val	Gly	Asn	Gly	Val	Ala	Val	Arg	Ser	Ser	Gln	Asn	Asn	Ile	Cys	Asp

Figure 3 Amino acid sequence of the regions of the RYR1 protein and related isoforms flanking the residue Arg614.[5 18 24–27] Residues identical to the human are boxed. Ala = Alanine, Arg = arginine, Asn = asparagine, Asp = aspartic acid, Cys = cystine, Gln = glutamine, Glu = glutamic acid, Gly = glycine, His = histidine, Ile = isoleucine, Leu = leucine, Ser = serine, Thr = threonine and Val = valine.

stimulators of channel opening such as halothane and caffeine. However, the sample number of this study was small and a larger sample size is necessary to verify this observation.

The clinical details of MHS probands carrying the Arg614Leu and Arg614Cys mutations are presented in table 2. The MH clinical grading scale devised by Larach and colleagues[23] was applied to each individual. As all the clinical indicators (clinical signs and laboratory tests) were not recorded or performed in each case during the anaesthetic reaction, direct comparison of the MH raw scores of probands was not possible (table 2). In addition, early diagnosis of an MH episode followed by prompt treatment implies that many of the clinical signs of an MH episode do not occur and a low raw score would thus result. This severely restricts the usefulness of the clinical grading scale for phenotype–genotype correlation studies. As seen in table 2, the phenotype of both the Arg614Leu and Arg614Cys mutations is variable: the presence of several clinical signs of the MH episode, such as muscular rigidity, respiratory acidosis, temperature increase.

Detection of the Arg614Leu mutation brings to nine the total number of RYR1 mutations reported to date in MHS/CCD pedigrees. These mutations cluster in two regions of the protein: seven mutations in the amino terminal region (residues Arg163 to Arg614) and two mutations near the centre of the protein (Gly2433 and Arg2434). Arg614 is the first amino acid of the RYR1 protein where two potentially causative mutations for MH are described.

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Since submission of this manuscript, two additional mutations in the RYR1 gene have been reported in MH.[31 32]

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