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ARTICLE

Recent advances in the diagnosis of malignant hyperthermia susceptibility: How confident can we be of genetic testing?

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Malignant hyperthermia (MH) is a condition that manifests in susceptible individuals only on exposure to certain anaesthetic agents. Although genetically heterogeneous, mutations in the RYR1 gene (19q13.1) are associated with the majority of reported MH cases. Guidelines for the genetic diagnosis for MH susceptibility have recently been introduced by the European MH Group (EMHG). These are designed to supplement the muscle biopsy testing procedure, the *in vitro* contracture test (IVCT), which has been the only means of patient screening for the last 30 years and which remains the method for definitive diagnosis in suspected probands. Discordance observed in some families between IVCT phenotype and susceptibility locus genotype could limit the confidence in genetic diagnosis. We have therefore assessed the prevalence of 15 *RYR1* mutations currently used in the genetic diagnosis of MH in a sample of over 500 unrelated European MH susceptible individuals and have recorded the frequency of *RYR1* genotype/IVCT phenotype discordance. *RYR1* mutations were detected in up to ~30% of families investigated. Phenotype/genotype discordance in a single individual was observed in 10 out of 196 mutation-positive

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families. In five families a mutation-positive/IVCT-negative individual was observed, and in the other five families a mutation-negative/IVCT-positive individual was observed. These data represent the most comprehensive assessment of *RYR1* mutation prevalence and genotype/phenotype correlation analysis and highlight the possible limitations of MH screening methods. The implications for genetic diagnosis are discussed.

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Introduction

Malignant hyperthermia (MH) is a pharmacogenetic disorder of skeletal muscle calcium regulation.[1] On exposure to certain anaesthetic agents (halogenated inhalational anaesthetics and depolarising muscle relaxants) susceptible patients may experience a range of symptoms, including skeletal muscle rigidity (from masseter spasms to generalised rigidity), metabolic acidosis, tachycardia and fever, as a consequence of an abnormally high release of intracellular calcium in skeletal muscle. The condition is potentially life threatening and is one of the main causes of morbidity and mortality during general anaesthesia.[2]

For the last 30 years the only reliable means of assessing an individual's risk from developing MH by avoiding exposure to triggering anaesthetic agents, has been via an invasive procedure, the *in vitro* muscle contracture test (IVCT).[3,4] The test requires a sample of patient skeletal muscle tissue, which is exposed *in vitro* to incremental doses of specific testing agents and the contracture response measured. One of the three laboratory diagnoses is possible: MH susceptible (MHS), MH normal (MHN) or MH equivocal (MHE). Patients classified as MHS and MHE are considered clinically at risk to MH, although the exact correlation between MHS/MHE IVCT phenotype and clinical development of an MH crisis is unknown. The IVCT has been standardised across Europe by the European Malignant Hyperthermia Group (EMHG) and shows a high degree of sensitivity (99.0%) and specificity (93.6%).[5] However, it is time consuming and expensive to perform and leaves patients with a small scar.

Genetically, MH susceptibility characterised by IVCT phenotype exhibits an autosomal dominant mode of inheritance with estimated prevalence of one in 8500.[2] The condition demonstrates both locus and allelic heterogeneity,[6,7] with six loci so far implicated of which four have been identified only in isolated families across Europe.[8–12] However, one locus on chromosome 19q13.1, the ryanodine receptor gene (*RYR1*), accounts for the majority of MH susceptible cases with $>50\%$ of families demonstrating linkage to this region.[13]

A long-term goal of the EMHG has been to assess whether DNA-based diagnosis for the condition is feasible. For several reasons, this has proven difficult as (1) the extent of genetic heterogeneity for MH susceptibility was not well established, (2) defects in the *RYR1* gene, the major susceptibility locus, were not widely characterised, (3) the majority of 'mutations' identified in the gene were missense, which made it difficult to predict the true effect of such variants on the functioning of the ryanodine receptor channel and (4) genotype–phenotype correlation analysis in MH susceptible families revealed discrepancies between IVCT phenotypes and *RYR1* genotypes, which further compounded the confusion as to the pathogenicity of *RYR1* variants identified.[14–16]

Following 10 years of genetic research and investigation of MH-susceptible families as a European consortium, a stage has now been reached where genetic screening for the condition can be offered in a limited capacity.[17] Genetic testing guidelines have been recently published by the EMHG to enable DNA diagnosis of MH in supplementation to the IVCT method of patient screening in MH families. In summary, genetic testing using certain mutations in the *RYR1* gene demonstrated to be 'causative' of MH through *in vitro* biochemical assays,[18,19] or through linkage analysis with markers flanking the *RYR1* locus is now feasible. Individuals carrying a 'causative' mutation or high-risk susceptibility haplotype are considered at risk of developing MH independent of IVCT diagnoses.

A main concern of introducing DNA diagnosis for the condition is the apparent discordance between IVCT phenotype and *RYR1* genotype in certain MH-susceptible families. Examples include patients classified as MHS by the IVCT but where the familial mutation or high-risk susceptibility haplotype is absent. In contrast, some cases classified as MHN by the IVCT have been reported to carry the familial mutation or high-risk susceptibility haplotype. Such individuals, under current testing strategies are considered at risk for developing MH on the basis of genetic data alone. False-negative diagnoses are more likely to have potentially fatal consequences under anaesthesia and are considered the most significant. To minimise false-negative results through genetic diagnosis and formally exclude risk from MH, the accepted practice of the EMHG is to use the IVCT for the characterisation of

individuals where the familial mutation, or high-risk haplotype is absent. The IVCT also remains the definitive method of diagnosis for the assessment of suspected MH probands.

Here, we have assessed the incidence of phenotype/genotype discordance across European MH families. We have collated data on 15 *RYR1* missense mutations described in the genetic testing guidelines. The data represent investigations by 11 European MH groups. The results give the most comprehensive assessment of the prevalence of these mutations across European populations, providing the most thorough analysis of the concordance between clinical (IVCT) phenotype and *RYR1* genotype to date and substantiate the rationale of the current DNA testing guidelines.

Materials and methods

Patients were all characterised using the European IVCT protocol. Prior to testing, the electrically invoked twitch (g) of the muscle specimen is recorded as an indicator of specimen viability. An MHS IVCT phenotype is diagnosed on the basis of an at least 0.2/g contracture on application of 2% halothane, and in an independent test on application of 2 mM caffeine. Individuals classified as MHE present a positive response to one of the trigger agents only. Normal individuals (MHN) do not react at the threshold dose of either trigger agent to give a sustained contracture of 0.2 g or above.

Genotypic data were collated from each contributing centre concerning the screening for the 15 *RYR1* mutations described in the current genetic testing guidelines. These have all been described previously.[20–33]

Results

Prevalence of *RYR1* mutations in Europe

Table 1 summarises the prevalence data for the 15 mutations from the screening of unrelated IVCT MHS individuals from Belgium, Italy, France, Germany, Switzerland and the UK. Although not all families were screened for all 15 mutations, three mutations appear to be prevalent in the European population, 1021G>A, 1840C>T, 7300G>A. These mutations account for 14.8, 28.6, and 34.2% of the mutation positive cases ($n=196$), respectively. Table 2 shows the relative contribution of data and prevalence of mutations in the participating groups. In France, Germany and Switzerland the 1840C>T mutation appears most prevalent, whereas in the UK the 7300G>A mutation accounts for the majority of mutation-positive cases. Where a uniform number of individuals were screened the mutation detection rate was 31.6% in France, 12.0% in Switzerland and 27.0% in the UK.

Discordance between *RYR1* genotype and IVCT phenotype

On segregation analysis of *RYR1* mutation with IVCT phenotype in the 196 mutation-positive families, 16 (8.0%) demonstrated discordance between the IVCT phenotype and *RYR1* genotype. In eight families the familial mutation was not detected in an IVCT MHS individual, and in the remaining eight, the familial mutation was detected in an IVCT MHN individual. Of the 15 mutations three (1021G>A, 1840C>T, 7300G>A) demonstrated phenotype/genotype discordance. These were the most prevalent mutations in the sample.

The IVCT data were re-examined in the 16 families showing discordant results. Criteria used for re-evaluation of the data in all family members included: (a) assessment

Table 1 Prevalence of *RYR1* mutations and frequency of *RYR1* genotype-IVCT phenotype discordance in Europe

RYR1 Exon	*Mutation nucleotide*	*Codon*	*Total no. of families*	*Prevalence*	*No of families discordant*	*No. families with an MHS individual without a mutation*	*No. families with an MHN individual with a mutation*
2	103T>C	Cys35Arg	569	1 (0.2%)			
6	487C>T	Arg163Cys	673	10 (1.5%)			
9	742G>A	Gly248Arg	587	1 (0.2%)			
11	1021G>A	Gly341Arg	737	29 (3.9%)	4	3	1
12	1209C>G	Ile403Met	594	0			
14	1565A>C	Tyr522Ser	574	1 (1.7%)			
15	1654C>T	Arg552Trp	569	0			
17	1840C>T	Arg614Cys	806	56 (6.9%)	7	4	3
17	1841G>T	Arg614Leu	607	4 (0.4%)			
39	6487C>T	Arg2163Cys	644	2 (0.3%)			
39	6488G>A	Arg2163His	610	4 (0.7%)			
45	7300G>A	Gly2434Arg	717	67 (9.3%)	5	1	4
45	7304G>A	Arg2435His	669	14 (2.1%)			
46	7372C>T	Arg2458Cys	610	5 (0.8%)			
46	7373G>A	Arg2458His	610	2 (0.3%)			
Total				196	16	8	8

Table 2 Prevalence of *RYR1* mutations across European populations

	Belgium		*Italy*		*France*		*Germany*		*Switzerland*		*UK*	
Mutation nucleotide	*No. F*	*Prev.*	*No. F*	*Prev.*	*No. F*	*Prev.*	*No. F*	*Prev.*	*No. F*	*Prev.*	*No. F*	*Prev.*
103T>C	NT	NT	NT	NT	106	1 (0.9%)	100	0	66	0	297	0
487C>T	17	0	48	2 (4%)	106	2 (1.9%)	139	2 (1.4%)	66	0	297	4 (1.3%)
742G>A	15	0	3	0	106		100	0	66	0	297	1(0.3%)
1021G>A	24	4 (16%)	42	0	106	9 (8.5%)	202	3 (1.4%)	66	1 (1.5%)	297	12 (3%)
1209C>G	17	0	8	0	106		100		66	0	297	0
1565A>C	3	0	2	0	106	1 (0.9%)	100		66	0	297	0
1654C>T	NT	NT	NT	NT	106		100		66	0	297	0
1840C>T	NT	NT	50	4 (8%)	106	12 (11%)	287	32 (11%)	66	3 (4.5%)	297	4 (1.3%)
1841G>T	NT	NT	38	0	106	3 (2.8%)	100	1 (1%)	66	0	297	0
6487C>T	11	1 (9%)	42	0	106		100	1 (1%)	66	0	297	1 (0.3%)
6488G>A	NT	NT	42	0	106		100		66	0	297	4 (1.3%)
7300G>A	NT	NT	41	1 (2.4%)	106	3 (2.8%)	207	7 (3.4%)	66	2 (3%)	297	53 (17.5%)
7304G>A	17	0	44	2 (4.5%)	106	1 (0.9%)	139	8 (5.8%)	66	0	297	5 (2%)
7372C>T	NT	NT	41	3 (7.3%)	106		139	2 (1.4%)	66	2 (3%)	297	0
7373G>A	NT	NT	41	0	106	2 (1.9%)	100	1 (1%)	66	0	297	0

No. F=number of families, Prev.=prevalence.

of the viability of the muscle specimen prior to testing, with good viability indicated by a pre-test twitch of 1 g or above, (b) reassessment of all IVCT trace measurements and (c) whether the methodology used for the IVCT conformed to the EMHG IVCT guidelines. In all discordant cases genetic data were repeated on an independent DNA sample where possible, to exclude the possibility of sample mix-up. In 10/16 families, discordance could not be explained by possible erroneous clinical or genetic data. In all 10 families, discordant phenotype/genotype data was observed in one individual. In five of these families an IVCT MHS individual did not carry the familial mutation and in five families a mutation was detected in an IVCT MHN individual. For these 10 families, of 48 IVCT-and DNA-tested individuals, 5/48 (10.4%) were discordant MHS (dMHS) and 5/48 (10.4%) were discordant MHN (dMHN) cases. By mutation type, of the total number of IVCT-tested individuals, 1021G>A demonstrated 2/23 dMHS, and 1/23 dMHN; 1840C>T demonstrated 2/20 dMHS and 2/20 dMHN, and 7300 G>A demonstrated 1/8 dMHS and 2/8 dMHN individuals (Table 3). MHE classified individuals are also considered clinically at risk to MH and may be equated with overall weaker contractures in the IVCT, or high responders to one specific testing agent used in the test. Discordance was only observed for mutation 1021G>A, where two MHE cases did not carry the familial mutation.

In the remaining six families, it was possible that the observed discordance was attributable to (a) clinical data that did not conform to current IVCT testing criteria having been recorded prior to European standardisation of the method (LMH03), (b) poor muscle specimen viability, indicated by a low pretest twitch response prior to the IVCT, resulting in potentially false-negative IVCT diagnoses (LMH52), c) DNA sample mix-up (LMH54) or (d) border-line MHS/ MHN IVCT contracture measurements (LZB, ULM31). In one family, no clinical data were available on the discordant individual (PAMH3) (Table 3).

Discussion

In the largest retrospective study of MH clinical and genetic data reported, we have shown that the relative prevalence of *RYR1* mutations varies in IVCT MHS patients across Europe inferring different founder events. The overall mutation detection rate between participating centres screening a uniform sample for all 15 mutations suggest that further mutations in *RYR1*, or mutations at other loci remain to be characterised. The data indicate that DNA testing for MH susceptibility in its present capacity will benefit up to 30% of MH families, depending on the population investigated, and provide the rationale for the development of mutation testing strategies.

The observed discordance between *RYR1* genotype and IVCT phenotype is a concern from the perspective of assigning the correct diagnosis of MH status. The IVCT is conducted to achieve high sensitivity at the cost of reduced specificity, thus discordance between phenotypic and genetic data is not unexpected. The presence or absence of a mutation can be considered more definitive in the genetic diagnosis of dominant Mendelian disorders. However, current genetic diagnostic strategies for assessing MH status are conservative in this respect because of the fact that (1) the majority of mutations associated with MH susceptibility are missense, where the pathogenicity of such mutations requires careful evaluation and (2) there is evidence that additional loci/mutations may contribute to MH susceptibility in individual families, which may

Table 3 European MH families demonstrating discordance between IVCT phenotype and *RYR1* genotype

			IVCT MHS individuals		*IVCT MHE individuals*		*IVCT MHN individuals*	
	Mutation	*No. individuals per family*	*No. with a mutation*	*No. without a mutation*	*No. with a mutation*	*No. without a mutation*	*No. with a mutation*	*No. without a mutation*
Gly341Arg 1021G>A	WZ76	13	3	1	0	1	0	8
	LMH53	5	3	0	0	0	1	1
	LMH03	**15**	**6**	**3**	**0**	**2**	**0**	**4**
	LMH55	5	1	1	0	1	0	2
Arg614Cys 1840C>T	BS012	3	1	1	0	0	0	1
	NA1	7	5	0	0	0	1	1
	WZ54	5	4	0	0	0	1	0
	*WZ11	5	4	1	0	0	0	0
	UL31	**3**	**1**	**0**	**0**	**0**	**1**	**1**
	PAMH3	**9**	**4**	**1**	**1**	**0**	**0**	**3**
	LZB	**40**	**19**	**2**	**1**	**7**	**0**	**11**
Gly2434Arg 7300G>A	UL129	3	2	1	0	0	0	0
	LMH52	**6**	**2**	**0**	**2**	**0**	**1**	**1**
	LMH54	**8**	**4**	**1**	**1**	**0**	**1**	**1**
	LMH56	3	1	0	0	0	1	1
	LMH57	2	0	0	1	0	1	0

Discordant families excluded from analysis owing to: early clinical data: one family; poor twitch data: three families; DNA sample mix-up: one family; threshold contracture: one family; no IVCT (in bold) data provided: one family.

account for or modify the observed IVCT response. Therefore, genetic testing guidelines are designed to minimise the possibility of a potentially fatal false-negative MH diagnosis in an individual that does not carry a mutation detected in other family relatives. Considering the IVCT as the bench mark for an MH-susceptible diagnosis, the frequency of such diagnoses occurs in 2.6% (5/190) of families or ~10% of individuals tested. Conversely, 2.6% (5/190) of families or ~10% of individuals demonstrate false-positive MH diagnoses, that is dMHN, on the basis of genetic data that could reflect variability in the IVCT. The discordance rates are heavily influenced by the number of individuals in each family who have been investigated by both IVCT and DNA methods. Unfortunately, genotype/phenotype concordance data were not available on all individuals from all mutation-positive families identified ($n = 196$). It is therefore possible that the values of 10% dMHS and 10% dMHN (95% CI 2–19 %) individuals are inflated. As the criteria used for the IVCT are designed to minimise false-negative MH diagnoses, the expectation was for the discordance rate to be greater in IVCT MHS, compared to MHN cases. However, inclusion of IVCT MHE data as a clinically at-risk group slightly elevates the frequency of discordance observed among individuals compared to the MHN group. Following the exclusion of potentially erroneous data from the analysis, and since all mutations studied have been demonstrated to confer some functional effect on the RYR1 protein through *in vitro* biochemical assays, mutation penetrance, genetic background, environmental effects and variability in the IVCT itself are likely to contribute to the observed discordance.[5,34,35]. In support of environmental effects, it has been well documented that the clinical penetrance of MH is varied, patients undergoing several uneventful anaesthetics before experiencing a reaction.

Only mutations 1021G>A, 1840C>T and 7300G>A demonstrated phenotype/genotype discordance. This observation may simply reflect the relative mutation prevalence. However, it has been shown that IVCT contracture response varies with mutation genotype. Those mutations associated with weaker contractures may therefore be expected to show higher discordance rates than those associated with more severe contracture phenotypes. All three mutations have been shown to be associated with weaker contractures compared to 487C>T, 6487C>T, 6488G>A and 7304G>A by analysis of channel mutants *in vitro*[18] and by comparative analysis of IVCT data in mutation carriers.[36,37]

With over 500 individuals investigated for *RYR1* mutations currently used in genetic diagnosis, this represents the most comprehensive evaluation of mutation prevalence and genotype/phenotype concordance data. We have shown that in 2.6% of families DNA tested, an individual may carry the familial *RYR1* mutation, where IVCT diagnosis would be negative. This finding should be considered when patients receive counselling on receipt of DNA test results. Conversely, the fact that in

2.6% of families an individual may be classified 'normal' by genetic diagnosis, when in fact IVCT MHS, supports the rationale of the current DNA testing guidelines to biopsy and IVCT to all individuals found not to carry a familial mutation or high-risk susceptibility haplotype. More importantly, the data emphasise that DNA screening for MH susceptibility is not feasible in isolation of the IVCT because of the risk, however small, of false-negative diagnoses.

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