Epigenetic allele silencing and variable penetrance of malignant hyperthermia susceptibility

R. L. Robinson¹, D. Carpenter¹, P. J. Halsall¹, D. E. Iles², P. Booms¹ ², D. Steele³, P. M. Hopkins¹*† and M.-A. Shaw²†

¹MH Investigation Unit, Academic Unit of Anaesthesia, St James’s University Hospital, Leeds LS9 7TF, UK
²Institute of Integrative and Comparative Biology, Faculty of Biological Sciences, L C Miall Building and
³Institute of Membrane and Systems Biology, Faculty of Biological Sciences, University of Leeds,
Leeds LS2 9JT, UK

*Corresponding author. E-mail: p.m.hopkins@leeds.ac.uk

Background. Tissue-specific monoallelic silencing of the RYR1 gene has been proposed as an explanation for variable penetrance of dominant RYR1 mutations in malignant hyperthermia (MH). We examined the hypothesis that monoallelic silencing could explain the inheritance of an MH discordant phenotype in some instances.

Methods. We analysed parent–offspring transmission data from MH kindreds to assess whether there was any deviation from the expected autosomal dominant Mendelian inheritance pattern. We also evaluated informative single-nucleotide polymorphism (SNP) genotypes in a cohort of unrelated MH patients using genomic DNA (gDNA, prepared from leucocytes) and coding DNA (cDNA, prepared from skeletal muscle). Finally, we examined the segregation of specific mutations at the gDNA and cDNA level within MH families where positive RYR1 gDNA genotype/normal MH phenotype discordance had been observed.

Results. In 2113 transmissions from affected parents, there was a consistent parent-of-origin effect (P<0.001) with affected fathers having fewer affected daughters (20%, 95% CI 17–22%) than affected sons (25%, 95% CI 23–26%) or unaffected daughters (27%, 95% CI 25–30%). No discrepancies were observed between the RYR1 SNP genotypes recorded at the gDNA and cDNA levels. In 14 MH negative individuals from 11 discordant families, the familial mutation was detected in skeletal muscle cDNA in all cases.

Conclusions. Epigenetic allele silencing may play a role in the inheritance of MH susceptibility, but this is unlikely to involve silencing of RYR1.


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The RYR1 gene on chromosome 19q13.1 encodes the skeletal muscle isoform sarcoplasmic reticulum calcium release channel (ryanodine receptor protein). RYR1 mutations have been detected in association with malignant hyperthermia (MH) and the rarer congenital myopathies central core disease and multiminicore disease. Certain RYR1 mutations produce both MH and central core disease, whereas others result in an MH phenotype only, a central core disease phenotype only, or in rare cases a multiminicore disease phenotype.¹⁻³ RYR1 mutations account for the majority of MH cases in the UK.⁴ RYR1 mutations detected in central core disease patients are in most cases dominantly inherited, although there are now several reports of apparently recessive RYR1 mutations in central core disease and multiminicore disease families.⁵⁻⁹ Since it was first linked to MH in 1990,¹⁰ more than 150 missense variants have been identified in RYR1.⁴ In 2001, guidelines for the use of specific RYR1 mutations in the diagnosis of MH risk were published.¹¹ These require

¹These authors contributed equally to this work.
confirmation of MH status using muscle biopsy and \textit{in vitro} contracture tests (IVCT) in cases where a familial mutation is not found. This is because MH families have been characterized where the familial $RYR1$ mutation is not always concordant with the MH phenotype, that is, there are MH-susceptible cases where the familial mutation is absent and MH normal cases where the familial mutation is present.\textsuperscript{12} Several explanations for this observation have been proposed, one being that additional loci/mutations may contribute to MH susceptibility in individual families which may account for or modify the observed clinical response.\textsuperscript{13} Mutation penetrance in relation to the clinical MH reaction and the IVCT assay used for clinical assessment could be influenced by environmental effects and variability in the IVCT itself and also epigenetic effects.

Epigenetic effects are any effects, other than DNA sequence, that alter the expression of genes and one such effect is allele silencing. For the vast majority of autosomal genes, the expression of both parental alleles is functionally equivalent. For some genes, however, the expression of the allele from one parent can be silenced, and thus called allele silencing or genomic imprinting. This genomic imprinting appears to occur in the gamete principally by epigenetic mechanisms, that is, without changing the DNA coding sequence. There are at least 70 imprinted genes known in mice and around 40 reported in humans.\textsuperscript{14} There is also evidence that allelic silencing may be tissue-specific, polymorphic (only occurring in the presence of specific polymorphic variants), or incomplete.\textsuperscript{15}

Until recently, it was not thought that $RYR1$ was subject to genomic imprinting; however, Zhou and colleagues\textsuperscript{8} reported the first evidence of $RYR1$ allelic silencing in a cohort of 11 families in which core myopathies were inherited in an apparently autosomal recessive fashion. In these cases, only the paternal allele carrying the mutation was transcribed, whereas the wild-type maternal allele was silenced at the transcriptional level (monoallelic expression). Further analyses of normal fetal tissues indicated that $RYR1$ allele silencing was also tissue-specific, polymorphic during early development, and likely to be developmentally regulated, as monoallelic expression was absent in normal adult skeletal muscle. It was suggested that long-range methylation effects could be responsible\textsuperscript{8} and that this phenomenon could also explain variable penetrance of dominant $RYR1$ mutations and thus the phenotype--$RYR1$ genotype discordance in MH.

To investigate the possibility of $RYR1$ silencing in MH, we evaluated transmission data within a large number of independent MH families, as any bias in transmission of MH susceptibility status could indicate an unusual mode of inheritance. To determine if tissue (skeletal muscle) specific allele silencing of $RYR1$ is a common feature of MH susceptibility, we sought differences in the allele frequencies in the coding DNA (cDNA) sequence compared with genomic DNA (gDNA) of numerous $RYR1$ single-nucleotide polymorphisms (SNPs) in unrelated MH patients, where gDNA extracted from blood and cDNA prepared from skeletal muscle RNA were available. Finally, we evaluated whether silencing of mutant alleles at the level of transcription in muscle, indicated by an absence of a mutation in the cDNA from muscle, could explain a normal muscle contracture response (IVCT) in patients in whom a mutation was detected on analysis of gDNA from blood.

\section*{Methods}

\subsection*{Patients and samples}

Patients contributing IVCT data attended the UK MH Investigation Unit in Leeds for diagnostic muscle biopsy and family studies between 1983 (adoption of the European MH Group consensus IVCT protocol) and 2007. In addition, those attending the Unit for diagnostic testing from 1990 onwards have been invited to contribute blood and muscle samples for research into the genetics of MH. These studies have received approval from the Local Research Ethics Committee and all participants have provided consent, which included consent for the retention of tissue and genetic material for use in future genetic studies of MH.

\subsection*{Definition of the MH phenotype}

Susceptibility status of suspected MH patients is assessed by the IVCT. Skeletal muscle biopsy specimens are exposed \textit{in vitro} to incremental concentrations of the trigger agents, halothane, and caffeine, and the contracture response of the muscle is measured. Specific changes in tension at threshold concentrations of the agents are used to define the IVCT phenotypes MHS (responds to both triggers, MH susceptible), MHE (responds to one trigger only, MH equivocal), and MHN (does not respond to either trigger, MH normal). The MHS and MHE IVCT phenotypes are both considered to indicate a high risk of developing MH clinically and as such the IVCT has a reported sensitivity and specificity of 99.0\% and 93.6\%, respectively.\textsuperscript{16}

\subsection*{Analysis of MH phenotype transmission data}

Transmission of the MHS, MHE, or MHN phenotype was investigated in all independent UK MH families with at least one confirmed case of clinical MH and where IVCT phenotypes were available for more than one generation ($n=537$). Statistical comparisons, using data from up to 2585 transmissions, were performed to determine if there was any evidence to suggest a bias in transmission of MH susceptibility between affected parents and children. We use the term ‘affected’ to define groups containing both MHS and MHE individuals. MHS individuals were also analysed separately and are defined as such.
Analysis of RYR1 SNP data from blood and skeletal muscle of unrelated MH-susceptible patients

Total RNA was prepared from frozen skeletal muscle biopsy specimens of 112 unrelated MHS patients using an ABI 6100 nucleic acid prep station according to the manufacturer’s instructions. These patients represented all those families who were previously genetically uncharacterized and for whom sufficient skeletal muscle tissue was available. cDNA prepared from the total RNA was used to sequence the ~15 kb RYR1 cDNA, using 25 overlapping PCR fragments of ~700 bp in length, read in both the forward and the reverse direction and analysed on an ABI 3730 (primer sequences and conditions available on request). gDNA extracted from whole blood was available for 62 of the 112 patients and this was genotyped, together with samples from additional unrelated MHS patients (for six SNPs) using custom-designed TaqMan® assays on an ABI 7900 platform according to the manufacturer’s protocol. All genotypes were anonymously determined before any comparison.

Analysis of skeletal muscle RYR1 transcripts in cases of genotype/phenotype discordance

As a matter of routine, when an RYR1 mutation is identified in a family, we screen for the familial mutation in other family members who have undergone the IVCT and for whom there is sufficient gDNA available. To date, we have identified RYR1 mutations in 284 UK MH families. For any individual with a normal IVCT result (MHN phenotype) but in whom the familial mutation was detected in gDNA, we initially re-evaluated the IVCT recordings to confirm specimens met the European MH Group viability criteria (www.emhg.org) and to check the accuracy of the original report. Where a discordant case of a positive genotype/normal IVCT phenotype in a patient without a core myopathy was confirmed (n=14), RNA was extracted from a muscle biopsy specimen as detailed above, cDNA synthesized, and the sample sequenced for the appropriate familial mutation. Histology reports for all these discordant individuals were also examined in order to exclude patients with core myopathies.

Data handling and statistical analyses

Data were entered onto Excel spreadsheets and summary data generated using functions within this software. Transmission data were analysed using $\chi^2$ tests with a continuity correction where appropriate using web-based software (URL: www.physics.csbsju.edu/cgi-bin/stats/contingency). Confidence intervals for proportions were generated assuming a binomial distribution. To assess the SNP data for consistency with the Hardy–Weinberg equilibrium, the significance of the difference between the observed genotype frequencies and those expected on the basis of the allele frequencies was calculated using HaploView v3.32 (URL: www.broad.mit.edu/mpg/haploview). Deviations from Hardy–Weinberg equilibrium can suggest inconsistencies in the genotyping assays as a result of mistyping of a particular genotype.

Results

Transmission of MH susceptibility within MH families

The numbers of sons and daughters (either affected or unaffected) born to affected parents were not significantly different ($P=0.138$, $\chi^2=2.19$, 1 df). Thus, a bias in transmission of sex to offspring of affected parents is excluded. We next proceeded to examine for parent-of-origin effects, that is, if an individual’s phenotype was influenced by whether it was their mother or father who was affected. Analysis of all 2585 transmissions indicated that 57% (95% CI 55–59%) of transmissions produced an affected offspring. This departure from the expected value of 50% might be a result of false positive diagnoses (inclusion of offspring with MHE phenotype) or because of selection bias through inclusion of the probands (index cases). Re-analysis after exclusion of all MHE cases indicated that 55% (95% CI 53–57%) of transmissions produced an affected offspring, suggesting that inclusion of the probands did indeed represent a significant selection bias. After exclusion of the probands, there was a total of 2113 transmissions, with 49% (95% CI 47–51%) producing an affected offspring.

However, there was a significant parent-of-origin effect with the children of affected mothers more likely to be affected than the children of affected fathers ($P<0.001$, $\chi^2=15.4$, 1 df, Table 1). This difference was reflected in the different proportions of affected children with affected fathers (45%, 95% CI 42–48%) compared with affected mothers (53%, 95% CI 50–56%). The parent-of-origin effect persisted when affected offspring were separated into MHS and MHE phenotypes ($P<0.001$, $\chi^2=16.1$, 2 df, Table 1). The significant parent-of-origin effect was still present when the affected and MHN offspring were separated into sons and daughters ($P<0.001$, $\chi^2=22.3$, 3 df, Table 2). From Table 2, it can be seen that the significant parent-of-origin effect is attributable to the reduced

<table>
<thead>
<tr>
<th>PhenoType of child</th>
<th>Affected father</th>
<th>Mother</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHS</td>
<td>337 (32%)</td>
<td>407 (39%)</td>
<td>744 (35%)</td>
</tr>
<tr>
<td>MHE</td>
<td>138 (13%)</td>
<td>148 (14%)</td>
<td>286 (14%)</td>
</tr>
<tr>
<td>MHN</td>
<td>592 (55%)</td>
<td>491 (47%)</td>
<td>1083 (51%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>1067</strong></td>
<td><strong>1046</strong></td>
<td><strong>2113</strong></td>
</tr>
</tbody>
</table>
Analysis of RYR1 SNP data from blood and skeletal muscle of unrelated MH-susceptible patients

No discrepancies were observed between the genotypes recorded at the gDNA and cDNA level (95% CI 0–6%). Of all possible genotypes, 27% were heterozygous. At least, 345 further gDNA SNP typings were available on additional unrelated MHS samples confirming the genotype frequencies observed were in Hardy–Weinberg equilibrium and that the SNP genotyping assays used were therefore robust and genotyping was accurate (Table 3).

Analysis of skeletal muscle RYR1 transcripts in cases of genotype/phenotype discordance

In 284 families with an identified RYR1 variant, 14 MHN individuals from 11 families had a familial variant detected in gDNA. Histological examination of all 14 patients excluded core myopathy phenotypes (Table 4). Two RYR1 mutations (c.742G>A and c.7300G>A), accounting for seven of the 11 familial variants, have been

Table 2 Transmission of MH susceptibility status to sons and daughters from affected parents. The affected individuals include MHS and MHE IVCT phenotypes. The values are numbers of individuals, with the percentage of the total in each column given in parentheses

<table>
<thead>
<tr>
<th>Phenotype of child</th>
<th>Affected parent</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Father</td>
<td>Mother</td>
</tr>
<tr>
<td>Affected son</td>
<td>264 (25%)</td>
<td>263 (25%)</td>
</tr>
<tr>
<td>Affected daughter</td>
<td>211 (20%)</td>
<td>292 (28%)</td>
</tr>
<tr>
<td>MHN son</td>
<td>301 (28%)</td>
<td>247 (24%)</td>
</tr>
<tr>
<td>MHN daughter</td>
<td>291 (27%)</td>
<td>244 (23%)</td>
</tr>
<tr>
<td>Total</td>
<td>1067</td>
<td>1046</td>
</tr>
</tbody>
</table>

Table 3 SNP analysis in RYR1 gDNA and cDNA in unrelated MH-susceptible patients. SNPs in bold were genotyped in the study described by Zhou and colleagues.8 HWE, Hardy–Weinberg equilibrium: P-value (significance of the difference between observed and expected genotype frequencies) calculated using HaploView v3.32 (URL: www.broad.mit.edu/mpg/haploview/)

Table 4 Details of families where a positive RYR1 genotype/normal IVCT phenotype discordance was observed. For all discordant patients, the IVCT contracture responses were 0 g at 2 mM caffeine and 2% (0.44 mM) halothane and the thresholds were therefore >2 mM caffeine and >2% halothane

<table>
<thead>
<tr>
<th>Family</th>
<th>Mutation</th>
<th>Familial MH susceptibility status (diagnosed by IVCT)</th>
<th>Parental origin of allele silencing to explain phenotype/genotype discordance</th>
<th>Histology report</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>c.742G&gt;A</td>
<td>3</td>
<td>1</td>
<td>Unable to deduce</td>
</tr>
<tr>
<td>2</td>
<td>c.3224G&gt;A</td>
<td>2</td>
<td>4</td>
<td>Maternal allele</td>
</tr>
<tr>
<td>3</td>
<td>c.7300G&gt;A</td>
<td>6</td>
<td>6</td>
<td>Paternal allele</td>
</tr>
<tr>
<td>4</td>
<td>c.7300G&gt;A</td>
<td>4</td>
<td>4</td>
<td>Unable to deduce</td>
</tr>
<tr>
<td>5</td>
<td>c.7300G&gt;A</td>
<td>1</td>
<td>4</td>
<td>Normal</td>
</tr>
<tr>
<td>6</td>
<td>c.7300G&gt;A</td>
<td>3</td>
<td>3</td>
<td>Maternal allele</td>
</tr>
<tr>
<td>7</td>
<td>c.7300G&gt;A</td>
<td>8</td>
<td>8</td>
<td>Maternal allele</td>
</tr>
<tr>
<td>8</td>
<td>c.7300G&gt;A</td>
<td>4</td>
<td>5</td>
<td>Paternal allele</td>
</tr>
<tr>
<td>9</td>
<td>c.11266C&gt;G</td>
<td>5</td>
<td>2</td>
<td>Maternal allele</td>
</tr>
<tr>
<td>10</td>
<td>c.14210G&gt;A</td>
<td>7</td>
<td>4</td>
<td>Maternal allele</td>
</tr>
<tr>
<td>11</td>
<td>c.14210G&gt;A</td>
<td>4</td>
<td>8</td>
<td>Maternal allele</td>
</tr>
</tbody>
</table>
functionally characterized using an in vitro system and shown to alter RYR1 calcium channel activity. The three variants (c.3224G>A, c.11266C>G, and c.14210G>A) which accounted for the remaining four individuals have not been detected on screening 200 unrelated normal control individuals and occur at conserved sites in RYR1, which is supporting evidence for these changes being functional mutations.

In three families, the parental origin of the mutation could not be deduced, due to lack of material and familial information. However, in six families, the mutant allele was of maternal origin and in two families, the mutant allele was of paternal origin. The familial mutation originally detected on analysis of gDNA was also detected on sequencing the cDNA in all 14 individuals investigated. Allelic silencing is therefore not responsible for the apparent reduced penetrance of dominant RYR1 mutations in these cases.

**Discussion**

We have used a large resource of phenotype and genotype data to systematically explore the possibility of epigenetic effects operating in the inheritance of the MH phenotype and whether any such effect may explain the variable penetrance of dominant RYR1 mutations in MH. We first assessed whether non-Mendelian transmission of the MH phenotype within families or a bias in transmission of susceptibility status between the sexes could indicate a more complex mode of inheritance and possible epigenetic parent-of-origin effects. We found the distribution of the numbers of sons and daughters from affected parents to be consistent with an autosomal mode of inheritance. However, our analyses of transmission of phenotype according to whether inheritance was from an affected father or mother produced consistent evidence for a parent-of-origin effect.

Our screening of family members for MH susceptibility is conducted in a systematic manner, but this is not randomized sampling and so there is the possibility of selection bias. Indeed, we found evidence that inclusion of probands contributed an obvious bias and we corrected for this, but we cannot exclude the possibility that further examples of sampling bias have influenced the results of our transmission analyses. Considering our transmission results at face value, the one consistent statistically significant finding appears to be a reduced transmission of MH susceptibility from affected fathers to their daughters. This is interesting because fewer females than males develop clinical MH, but Islander and colleagues reported a lower incidence of positive IVCT results in female vs male relatives of Swedish MH cases. If our finding represents a real epigenetic phenomenon, it could be explained by the silencing of a mutant allele on the X chromosome in the presence of a normal allele. This allele silencing could be polymorphic or the gene involved may be implicated in only a minority of MH cases.

Our study provides clear evidence, however, that RYR1 allelic silencing does not explain the known cases of RYR1 mutation/MHN IVCT phenotype discordance in the UK MH population. RYR1 allelic silencing would only explain apparent lack of penetrance of dominant mutations in MH families, if a mutation detected on analysis of gDNA from blood was not detected in the MHN individual’s skeletal muscle cDNA. We considered 11 families where at least one family member was present where the familial mutation was detected in gDNA but where the muscle biopsy was normal: we found the familial mutation in the skeletal muscle cDNA of all 14 discordant individuals.

In contrast, Zhou and colleagues reported silencing of the wild-type allele in core myopathy families, which enabled penetrance of apparently recessive RYR1 mutations when in the hemizygous state. In their study, six symptomatic core myopathy patients (from four sporadic and two familial cases) were all hemizygous for RYR1 mutations in skeletal muscle cDNA due to silencing of the maternal wild-type allele and heterozygous in gDNA. There was, however, no evidence for compound heterozygosity which would normally be indicative of recessive inheritance. Rather, a recessive model was proposed because the parents of the affected child in the two familial cases did not show symptomatic features of disease. This may be presumptuous as 40% of individuals demonstrating histological features of core myopathy are clinically asymptomatic.

Zhou and colleagues reported that RYR1 allele silencing also occurs in normal fetal tissues (n=39), where it is also tissue-specific. As it was only observed in 10% of cases, it was considered polymorphic during early development and developmentally regulated. This was assumed because monoallelic expression was absent on examination of normal adult skeletal muscle samples (n=25). To further evaluate these findings, as only a small number of adult muscle specimens were previously investigated, we examined six independent RYR1 SNP genotypes (Table 3). No discrepancies were observed between genotypes recorded at the gDNA and cDNA level, and thus exclusively biallelic expression of the RYR1 SNPs was observed in adult skeletal muscle samples. This observation provides further evidence that epigenetic modification of RYR1 expression is unlikely to play a role in the inheritance of MH susceptibility.

In conclusion, our data suggest a reduced penetrance of MH susceptibility in females. Our molecular data do not suggest that epigenetic RYR1 allele silencing is polymorphic in adult patient skeletal muscle, nor do we observe that RYR1 allelic silencing modifies the penetrance of ‘dominant’ RYR1 mutations detected in MH-susceptible families. These data indicate that monoallelic silencing of
**RyRI** is likely to be a rare event and possibly specific to patients with core myopathy phenotypes.

**Acknowledgement**

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**Funding**

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