Discordance, in a Malignant Hyperthermia Pedigree, between In Vitro Contracture-Test Phenotypes and Haplotypes for the MHS1 Region on Chromosome 19q12–13.2, Comprising the C1840T Transition in the RYR1 Gene

T. Deufel,1 R. Sudbrak,1 Y. Feist,3 B. Rübsam,4 I. Du Chesne,1 K. L. Schäfer,2 N. Roewer,5 T. Grimm,3 F. Lehmann-Horn,6 E. J. Hartung,4 and C. R. Müller3

1Stoffwechselabteilung, Klinik und Poliklinik für Kinderheilkunde (Allgemeine Kinderheilkunde), and 2Gerhard-Domagk-Institut für Pathologie, Universität Münster, Münster; 3Institut für Humangenetik and 4Institut für Anästhesiologie, Universität Würzburg, Würzburg; 5Abteilung für Anästhesiologie, Universitäts-Krankenhaus Eppendorf, Hamburg, and 6Abteilung Angewandte Physiologie, Universität Ulm, Ulm

Summary

A point mutation in the gene encoding the skeletal muscle calcium release channel (RYR1) has been proposed as the probable cause of malignant hyperthermia (MH) in swine, where it segregates with the disease in all MH-prone strains investigated. The same C-to-T exchange in nucleotide position 1840 of the human RYR1 cDNA sequence was found in a few human MH pedigrees. We report a German MH pedigree where in vitro contracture test (IVCT) results and haplotypes of markers for the MHS1/RYR1 region including this base transition have yielded several discrepancies. The MH-susceptible phenotype was defined by IVCT performed according to the European standard protocol. Haplotypes were constructed for markers for the MHS1/RYR1 region on chromosome 19 and include the C1840T base exchange. Discussing the probabilities for a number of hypotheses to explain these data, we suggest that our results may challenge the causative role of this mutation—and possibly the role of the RYR1 gene itself—in human MH susceptibility, at least in some cases.

Introduction

Malignant hyperthermia (MH) is a pharmacogenetic myopathy triggered by certain inhalational anesthetic agents (e.g., halothane) and depolarizing muscle relaxants (e.g., succinylcholine). Susceptibility to MH is inherited as an autosomal dominant trait with an estimated incidence in the range of 1:12,000–1:40,000 (Britt and Kalow 1970; Ellis and Halsall 1980; Ording 1985). Susceptible individuals may react with an MH crisis when exposed to a triggering agent, but otherwise they do not show any clinically relevant signs. The only way, therefore, to detect susceptibility to MH in probands at risk is by an in vitro contracture test (IVCT) performed on a fresh muscle sample obtained by biopsy. According to a standardized protocol for this test, as established by the European Malignant Hyperthermia Group (EMHG 1984), probands are assigned the status of MH susceptible (MHS), MH normal (MHN), and MH equivocal (MHE). An animal model, porcine MH, mimics most clinical, electrophysiological, and biochemical characteristics of MH in man, and it was on the basis of genetic data obtained in the pig, linking halothane sensitivity to the GPI locus on porcine chromosome 6, that a homologous MHS1 locus on human chromosome 19q12–13.2 was identified (McCarthy et al. 1990). More recent data, however, strongly suggest genetic heterogeneity of human MH, which has been excluded from the MHS1 locus in a number of pedigrees (Levitt et al. 1991; Deufel et al. 1992b; Fagerlund et al. 1992; Iles et al. 1992); two novel MHS loci have been mapped to chromosomes 7q (Iles et al. 1994) and 3q13.1 (Sudbrak et al. 1995).

The gene encoding the skeletal muscle sarcoplasmic reticulum calcium channel (ryanodine receptor; RYR1) has been cloned (Zorzato et al. 1990). It was mapped to chromosome 19q13.1 and was proposed as a candidate for the molecular defect in MH (MacLennan et al. 1990). A base transition in the RYR1 gene, C1840T, resulting in a substitution of cysteine for arginine in position 614 of the human ryanodine receptor protein, has been suggested as a candidate mutation causing MHS in patients where the disease maps to the MHS1/RYR1 locus on chromosome 19q13.1 (Gillard et al. 1991). Overwhelming support for this hypothesis comes from the animal model of MH in swine, where the corresponding mutation has been shown to cosegregate with the trait in >300 meioses (Fujii et al. 1991; Otsu et al. 1991). In humans, this was found in 2 of 50 pedigrees.
with MH (Gillard et al. 1991; Hogan et al. 1992; MacLennan and Phillips 1992). In our own investigation of 120 independent MH pedigrees, we found the mutation in 10 (Deufel et al. 1992a; Rübسام et al. 1993). The frequency of this mutation in humans with the MHS phenotype can thus be estimated as ranging from 5% to 10%. It should be noted that so far no individual has been described as carrying this mutation and showing the MHN phenotype in a MH pedigree. Furthermore, the mutation has been detected neither in any of the MH pedigrees where the MHS phenotype was excluded from the chromosome 19 MHS1 locus nor in several hundred control subjects investigated. We report a German pedigree with MH, where IVC testing and molecular genetic analyses produced discordant results that challenge current hypotheses on the molecular basis of MH susceptibility.

Material and Methods

Genomic DNA was prepared from EDTA whole blood and untreated slices of paraffin-embedded muscle tissue obtained at autopsy, according to methods described elsewhere (Kunkel et al. 1977; Miller et al. 1988). The microsatellite repeat markers for the loci D19S75, D19S191, RYR1, D19S190, and D19S47 were amplified by PCR, were radiolabeled, were separated on sequencing gels, and were typed as described elsewhere (Sudbrak et al. 1993). The C1840T base exchange in the RYR1 gene was analyzed by PCR of total genomic DNA, followed by digestion with Rsal and electrophoresis on 8% nondenaturing polyacrylamide gels. The primers used were those described in a later modification (Otsu et al. 1992) of the original assay (Gillard et al. 1991). Solid-phase sequencing was performed on single-stranded DNA from a 220-bp PCR product amplified using the forward primer, as above (Otsu et al. 1992), together with primer 2 from the original report (Gillard et al. 1991), which had been biotinylated for template preparation. Only for individual 506, this product, without prior biotinylation, was cloned into pBluescriptKS (Stratagene). Either the unbiotinylated reverse primer (primer 2), for direct sequencing, or M13 universal primer, for cloned PCR products from individual 506, was used for sequencing according to the dideoxy method with fluorescein–15–dATP (AutoRead; Pharmacia Biotech) as an internal label and with an automated laser fluorescence sequencing apparatus (A.L.F.; Pharmacia Biotech).

Two-point linkage analysis was performed using version 5.1 of the LINKAGE package of programs (Lathrop et al. 1984). Parameters were set as described elsewhere (Deufel et al. 1992b), except for those varied in the genetic models shown in table 1. The physical and genetic order of the markers was assumed to be as reported elsewhere (Iles et al. 1992; Mulley et al. 1993).

Results

IVCT for MH Susceptibility

The pedigree MH011 is shown in figure 1. The index patient (401) died as a consequence of a fulminant MH crisis precipitated by general anesthesia with halothane. This prompted investigation of the MH risk status in the pedigree by the IVCT, according to the European standard protocol (EMHG 1984). The test determines the threshold concentrations, of the triggering agents halothane and caffeine, at which a contracture force >0.2 p is provoked in freshly isolated muscle specimens obtained by biopsy. According to the protocol, a proband is typed MHS (susceptible), MHN (negative), or, in few cases, MHE (equivocal). The results are listed in table 2. The mother (302) and one sister (408) of the index case were MHS, and two brothers (403 and 405) were typed MHN. Two sons (501 and 503) of 401 were also MHS, and one daughter (506) was MHN. Individual 409 is a first-degree cousin of the index case and was found to be MHS, as were his two sons (511 and 513). His sister (412) is MHN, and their father (303) had died earlier without any findings suggesting MH and was not studied.

Markers for the MHS1/RYR1 Locus on Chromosome 19q

The pedigree was typed with polymorphic microsatellite markers for the loci D19S75, D19S191, RYR1, D19S190, and D19S47. The physical and genetic localization of these markers is known (Iles et al. 1992; Mulley et al. 1993), placing D19S75 and D19S191 proximal to, and D19S190 and D19S47 distal from, RYR. Unequivocal haplotypes can be constructed for most markers in all typed individuals. As shown in figure 1, however, the eight MHS individuals in this pedigree do not share a common haplotype. Individual 408 is MHS, and she has inherited the 3–6–2–1–6 haplotype from her affected mother (302). The second maternal haplotype, 2–5–4–2–2, is found in her two unaffected brothers (403 and 405). Both haplotypes of the deceased father (301) can be reconstructed, and one of them (1–1–2–2–6) is found in four MHS individuals (401, 408, 501, and 503), as well as in the MHN grand-daughter (506). Individual 409 is a first-degree cousin of the index case and was also typed MHS. He does not share a haplotype with his cousins 401 and 408 but shares both haplotypes with his sister (412), who is MHN. He furthermore transmits different haplotypes to his two sons (511 and 513), both typed as MHS. Linkage of the MHS trait, as defined by the IVCT, to markers from the MHS1/RYR1 region: therefore is only possible if one assumes a false-negative IVCT result for individual 506 in the left branch of the pedigree (fig. 1) while the right branch appears to be unlinked at all.
Table I

Likelihoods for Linkage of the IVCT Phenotype to Markers for the RYR1/MHS1 Locus on Chromosome 19q13.1

<table>
<thead>
<tr>
<th>Model</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>A 1:100</th>
<th>B 1:1,000</th>
<th>C 1:5,000</th>
<th>D 1:10,000</th>
<th>E 1:50,000</th>
<th>F 1:100,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 ......</td>
<td>.98</td>
<td>1.00</td>
<td>-5.40</td>
<td>-8.40</td>
<td>-10.50</td>
<td>-11.40</td>
<td>-13.50</td>
<td>-14.40</td>
</tr>
<tr>
<td>2 ......</td>
<td>.98</td>
<td>.98</td>
<td>-4.20</td>
<td>-4.50</td>
<td>-5.10</td>
<td>-5.10</td>
<td>-5.10</td>
<td>-4.90</td>
</tr>
<tr>
<td>3 ......</td>
<td>.98</td>
<td>.95</td>
<td>-3.70</td>
<td>-4.60</td>
<td>-4.80</td>
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<td>-1.00</td>
<td>-0.80</td>
<td>-0.30</td>
<td>-0.20</td>
</tr>
</tbody>
</table>

*Likelihoods were calculated for linkage of the MH phenotype as defined by the IVCT to the RYR1/MHS1 locus defined by a haplotype for the markers D19S191, RYR1-MS, RYR1-1840C→T, and D19S190 in the order given; for convenience, results are expressed as lod scores. The physical order of the markers has been reported elsewhere (for details, see Material and Methods).

C1840T Base Exchange in the RYRI Gene

The C1840T base exchange was analyzed by RsaI digestion of an amplified genomic DNA fragment (Otsu et al. 1992). The 922-bp product comprises, along with the one RsaI site abolished by the mutation, another two RsaI sites, which serve as an internal control for the restriction-enzyme digest. Four fragments—550, 199, 179, and 12 bp in length—are found after RsaI digestion of PCR products amplified from the DNA of nonaffected individuals, as well as from DNA of affected individuals who do not show the C1840T base exchange. Presence of the mutation—and thus loss of one RsaI site—is indicated by the appearance of a further fragment, of 729 bp (fig. 2). The results were confirmed by DNA sequencing of PCR products (fig. 3). The results of the mutation assay are included in figure 1, along with the microsatellite marker alleles for the MHS1 locus. It is evident that the C1840T base exchange is present on two different haplotypes in the pedigree. Individual 408 appears to be homozygous for the mutation and has received one C1840T allele each from her MHS mother (302) and her father (301). The latter was not available for testing by the IVCT but has no personal or family history of MH susceptibility. It is on this paternal haplotype that the mutation was transmitted to the index case 401 and to his three children (501, 503, and 506). No mutation was observed in the entire right branch (fig. 1) of the pedigree.

Variation of Genetic Models Used in Likelihood Estimates

Likelihoods for linkage of the MHS trait to the MHS1/RYR1 haplotype including the C1840T transition were calculated for the observed set of data, by employing different genetic models that account for potential inaccuracies with respect to ascertainment of the phenotype and mutation frequency. The parameters characterizing these models are listed in table 1. Models in lines 1–5 differ with respect to the assumed diagnostic performance of the IVCT, ranging from optimal conditions (model 1, sensitivity .98, specificity 1.0), i.e., a false-positive rate of zero, to poor performance (model...
Table 2
Results of IVCT

<table>
<thead>
<tr>
<th>INDIVIDUAL</th>
<th>CAFFEINE TEST</th>
<th>HALOTHANE TEST</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Threshold Concentration (p)</td>
<td>Force at Threshold (p)</td>
</tr>
<tr>
<td>------------</td>
<td>---------------</td>
<td>----------------</td>
</tr>
<tr>
<td>302</td>
<td>2.0</td>
<td>.2</td>
</tr>
<tr>
<td>403</td>
<td>3.0</td>
<td>.3</td>
</tr>
<tr>
<td>405</td>
<td>&gt;4.0</td>
<td>0</td>
</tr>
<tr>
<td>408</td>
<td>.5</td>
<td>.2</td>
</tr>
<tr>
<td>409</td>
<td>1.5</td>
<td>.2</td>
</tr>
<tr>
<td>412</td>
<td>3.0</td>
<td>.3</td>
</tr>
<tr>
<td>501</td>
<td>1.5</td>
<td>.7</td>
</tr>
<tr>
<td>503</td>
<td>2.0</td>
<td>.8</td>
</tr>
<tr>
<td>506</td>
<td>&gt;4.0</td>
<td>0</td>
</tr>
<tr>
<td>511</td>
<td>1.5</td>
<td>.3</td>
</tr>
<tr>
<td>513</td>
<td>1.5</td>
<td>.75</td>
</tr>
</tbody>
</table>

Note.—For individuals 302, 403, 405, 408, 409, and 412, IVCT was performed by F.L.-H.; for individuals 501, 503, and 506, IVCT was performed in Würzburg by E.J.H. and B.R.; and for individuals 511 and 513, IVCT was performed in Hamburg by N.R. ND = not determined.

5, sensitivity and specificity .90), i.e., 10% false-positive and 10% false-negative results. Models A–F differ with respect to the assumed gene frequency of the MHS trait, ranging from very high (A) to very low prevalence (F). Model 1D (2% false-negative rate, no false positives, gene frequency 1:10,000) corresponds to the consensus settings that were agreed on by the EMHG genetics sections and have been used in previously reported linkage studies on MHS (Deufel et al. 1992b; Iles et al. 1992, 1993; Sudbrak et al. 1993, 1995).

As shown in table 1, the hypothesis of linkage of the MHS trait to chromosome 19 in this pedigree (and thus a potential involvement of the RYR1 gene in its pathology) is rejected for all the genetic models except for model 5, where lod scores >-2.0 are obtained. Thus, the results obtained in the pedigree, on varying the performance characteristics of the IVCT, are compatible with linkage of MHS to the MHS1/RYR1 locus only if both specificity and sensitivity of the test are reduced to 90%. This holds true even for extremely high prevalence values and, thus, an increased probability for more than one MHS trait segregating in the pedigree.

Discussion

Two C1840T base transitions segregate in this pedigree, which are different by descent. One is transmitted from the grandmother, typed MHS, to her affected (i.e., MHS) daughter. A second mutation is present on the reconstructed grand–paternal haplotype inherited by several MHS and one MHN individuals in one part of the pedigree. The MHS phenotype in this part of the pedigree (fig. 1, left) could well be attributed to the C1840T transition, provided, however, that one false-negative IVCT result is assumed. To date, there is no published evidence for a true false-negative (i.e., MHN) result of the IVCT according to the European standard protocol (EMHG 1984; Ørting 1987, 1988; Ørting et al. 1991); the sensitivity of the test is considered to be

Figure 2 Detection of the C1840T base exchange in the RYR1 gene, by restriction-enzyme analysis. Amplification of genomic DNA by PCR was followed by separation on polyacrylamide gels, as described elsewhere (Otsu et al. 1992). Lanes M contain a DNA length marker (100-bp ladder; the lowest fragment shown is 100 bp); the undigested product is 922 bp in length, as shown in lane L. Digestion of the wild-type sequence containing two RsaI sites produces four fragments, 350, 199, 179, and 12 bp in length. The mutation C1840T results in the loss of one site, and an additional fragment, of 729 bp, is observed. The numbering of lanes containing digested product corresponds to that in fig. 1. Individual 408 is homozygous for the 729-bp fragment; the wild-type fragments are missing; individuals 403 and 409 are homozygous for the wild–type pattern, and individuals 501 and 506 are heterozygous for it.
Figure 3  Identification of the C1849T base exchange in the RYR1 gene, by sequence analysis. A, Direct sequencing of PCR-amplified DNA, showing individual 408 to be homozygous for the C-to-T exchange in position 1840 of the RYR1 gene. Results are shown for individuals 412 (MHN; wild-type sequence) (upper panel), 302 (MHS; C1840T heterozygous) (middle panel), and 408 (MHS; C1840T homozygous) (lower panel). Lettering below each graph refers to the base identified by the sequencer, “Y” denotes the simultaneous presence of both C and T (for details, see Material and Methods). B, Sequencing of cloned PCR product from individual 506 (MHN), showing equal numbers of clones with either wild-type (upper panel) or mutated (lower panel) sequence indicating heterozygosity for the C1840T base exchange (for details, see Material and Methods).
high. Furthermore, the IVCT data of this particular patient (table 2) do not indicate that hers is a false-negative result that could resolve the apparent discrepancy with mutation analysis. Nonpenetrance due to the influence of a hypothetical modifying gene effect might be one explanation.

Three MHS individuals in another branch of the pedigree (fig. 1, right) do not carry the C1840T transition; nor do they share with their affected relatives any of the associated haplotypes. In addition, two MHS individuals have received two different MHS1/RYR1 haplotypes from their MHS father, who again shares his genotype with an unaffected (i.e., MHN) sister. In these patients, therefore, the MHS mutation is most likely unrelated to the C1840T transition in the RYR1 gene and is unlinked to the MHS1/RYR1 locus.

One individual is homozygous for the mutation, which has been confirmed by direct sequencing (fig. 3A). Her MHS phenotype according to the IVCT is not different from that of the other MHS relatives either heterozygous for or not carrying the mutation (table 2). In view of both the frequency of the mutation observed among MHS patients by us and others and the presumed prevalence of the MHS phenotype as a whole, however, the occurrence of two independent mutations in a single pedigree is an unexpected finding.

Summarizing our mutation data, we note that two RYR1 C1840T mutations segregate independently in the pedigree and that the presence of yet a third mutation, unrelated to RYR1, is required to explain MHS in those individuals carrying neither of the two RYR1 mutations. This contrasts with available data on the frequency of MHS and on the frequency of the C1840T base exchange itself among patients with MHS linked to the MHS1 locus on chromosome 19 (Britt and Kalow 1970; Ellis and Halsall 1980; Ørding 1985; Gillard et al. 1991; MacLennan and Phillips 1992).

The pattern of inheritance of the MHS phenotype as defined by the IVCT in this pedigree is compatible with the segregation of a single autosomal dominant trait. Yet, the C1840T base transition is present only in some but not all of the MHS individuals. Thus, multiple recombination events between the MHS phenotype in this pedigree and the entire haplotype of chromosome 19q markers comprising the MHS1 and RYR1 loci have to be postulated if the C1840T mutation is indeed the cause of MHS in all affected individuals. All molecular data in this pedigree have been confirmed by repeated analysis and in most cases were obtained on two different DNA samples and can therefore be considered solid. The order of markers on chromosome 19, as well as their linkage to the MHS1/RYR1 locus, again is well established. In comparison, the reliability of the IVCT results is more difficult to assess. The same is true for epidemiological data concerning the prevalence of MHS and, therefore, the probability that there is segregation of more than one MHS mutation in the same pedigree. Both factors obviously may confound the results of linkage analysis. As our results in table 1 show, lod scores are very sensitive to MHS prevalence, with less negative values for very high prevalence, if the performance of the IVCT is assumed to be good (models 1A–1F). Even at a highly unlikely MHS prevalence of 1:100, however, linkage is firmly excluded with a lod score of −5.40. With poorer IVCT performance the influence of MHS prevalence on obtained lod scores decreases and is nearly lost (models 2–4). Varying influencing factors of linkage analysis to extreme values, with respect to both test performance and MHS prevalence, therefore does not significantly increase the probability that MHS is linked to the RYR1 locus in this pedigree where a presumed mutation in the RYR1 gene is present.

In conclusion, three hypotheses can alternatively explain the findings in this pedigree, and they must be evaluated on the basis of our current knowledge of the biochemistry, genetics, and epidemiology of MHS: (1) MHS is caused by two independent RYR1 mutations in one branch of the family and by a third mutation that is unrelated to RYR1 in the other branch; in addition, one false-negative IVCT result must have occurred in individual 506. (2) RYR1-C1840T mutations alone are the cause of MHS in the entire pedigree. Individual 506 then would still have to be a false-negative IVCT, while individuals 409, 511, and 513, in addition, must be false positives. (3) A single dominant mutation accounts for all observed MHS phenotypes in the family; this mutation then would most likely be unlinked to chromosome 19q13.1 and, thus, to the RYR1 locus.

At present, we cannot reject any one of these three explanations. As for the first hypothesis, it requires the simultaneous presence of three independent causative MHS mutations together with one false IVCT result and thus is in contrast with published estimates of the prevalence of both MH and the C1840T mutation (Britt and Kalow 1970; Ellis and Halsall 1980; Ørding 1985; Gillard et al. 1991; MacLennan and Phillips 1992). The extent to which these estimates would have to be corrected is reflected in the lod-score calculations shown in table 1. The second hypothesis can only be upheld, as demonstrated in table 1, if both specificity and sensitivity of the IVCT test are drastically lowered, and thus, again, the hypothesis is in disagreement with available data in the literature (EMHG 1984; Ørding 1987, 1988; Ørding et al. 1991). At present, these results, though obtained on small numbers of patients with an MH crisis, would not support this notion. Finally, the third hypothesis is compatible with the results shown in table 1 but seems in striking contrast to reported genetic and biochemical evidence, which implicates the C1840T mutation at least in the animal model of porcine MH (O'Brien 1986;
Mickelson et al. 1988; Fujii et al. 1991; Otsu et al. 1991). A very recent study, however, in a strain of pigs with MH, the first one to correlate established genotypes for the RYR1 mutation with the MH phenotype, does not detect an MHS phenotype in any of the animals that are heterozygous carriers of the mutation. Even homozygous animals have marked variations in the penetrance of various criteria defining the MHS phenotype. In their conclusions, the authors of that study postulate that presence of the C1840T mutation alone might not be sufficient to consistently cause MH in pigs (Fletcher et al. 1993). Furthermore, it should be taken into consideration that biochemical evidence for altered function of mutated ryanodine receptor has not been provided yet. In humans, the C1840T transition has only been demonstrated in nuclear families too small to allow testing of linkage with MHS; its being a polymorphic variation strongly associated with a MHS mutation in the RYR1 receptor in some if not most cases is thus still a viable assumption. We feel that our results raise important questions concerning the epidemiology of MH susceptibility and its ascertainment in probands. They also challenge the hypothesis of the C1840T base exchange being a mutation that by itself causes the MHS phenotype.

Acknowledgments

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