

# Presence of Two Different Genetic Traits in Malignant Hyperthermia Families

## Implication for Genetic Analysis, Diagnosis, and Incidence of Malignant Hyperthermia Susceptibility

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**Background:** Malignant hyperthermia susceptibility (MHS), an uncommon syndrome often inherited as an autosomal dominant trait, is characterized by a genetic and clinical heterogeneity. In this article, the authors described six pedigrees in which both parents of MHS patients were diagnosed with MHS by an *in vitro* diagnostic test. Haplotype and mutation analysis revealed that more than one MHS genetic trait was present in these families.

**Methods:** A panel of 104 MHS families were investigated with a caffeine halothane contracture test on muscle biopsy specimens. When possible, blood creatine kinase concentrations of MHS patients were measured. Haplotyping studies were conducted with chromosome 19q13.2 polymorphic markers and mutations were searched for in patients' DNA.

**Results:** In six families, the *in vitro* diagnostic test and genetic studies demonstrated that both, apparently unrelated, parents of MHS patients were MHS. In three families, homozygous or compound heterozygous individuals for RYR1 mutations were characterized at a molecular level. In one family, a compound heterozygous patient harboring a RYR1 mutation and a CACNA1S mutation was identified. While patients with two mutated alleles did not show differences in their muscle response to halothane or caffeine, their creatinine kinase concentrations were significantly elevated compared with the heterozygous patients.

**Conclusions:** Based on genetic and *in vitro* diagnostic test data, more than one MHS allele associated with the MHS phenotype was evidenced in four families. These data should be considered in view of the use of genetics for the diagnosis of MHS and when reaching conclusions of genetic heterogeneity in MHS families. Taking into account the usual dominant mode of transmission of MHS and the size of the investigated population, the authors propose an evaluation of the incidence of the MHS in the general population based on genetic data.

MALIGNANT hyperthermia (MH) is usually considered as an autosomal dominantly inherited and potentially

fatal disorder of skeletal muscle.<sup>1</sup> An MH episode can be triggered in susceptible individuals by exposure to halogenated inhalational anesthetics and depolarizing muscle relaxant during anesthesia.<sup>2</sup> Signs include skeletal muscle rigidity, metabolic acidosis, hyperkalemia, cardiac dysrhythmia, and hyperthermia.<sup>3</sup> The incidence of MH reactions is estimated at about 1 in 15,000 children and 1 in 50,000 adults undergoing anesthesia in North America and Europe.<sup>4</sup> Because of the subclinical nature of the condition, an *in vitro* diagnostic test (IVCT) for diagnosis of MH susceptibility (MHS) has been developed. This test is based on the differential contractile response of normal and MH muscle to halothane and caffeine. North American<sup>5</sup> and European<sup>6</sup> protocols have been established. The North American protocol identifies patients as MH normal or MHS, while patients investigated using the European protocol are diagnosed as MH normal, MHS, or MH equivocal (MHE). The clinical and biochemical relation between the MHS and MHE phenotypes is unclear. Because of the incomplete penetrance of the gene, the difficulty in defining mild reactions, and the caution and care now taken by anesthesiologists, it is rather difficult to determine the actual incidence of MHS genes in the general population. While an incidence value of 1 in 10,000 is usually admitted, this figure may be underestimated.

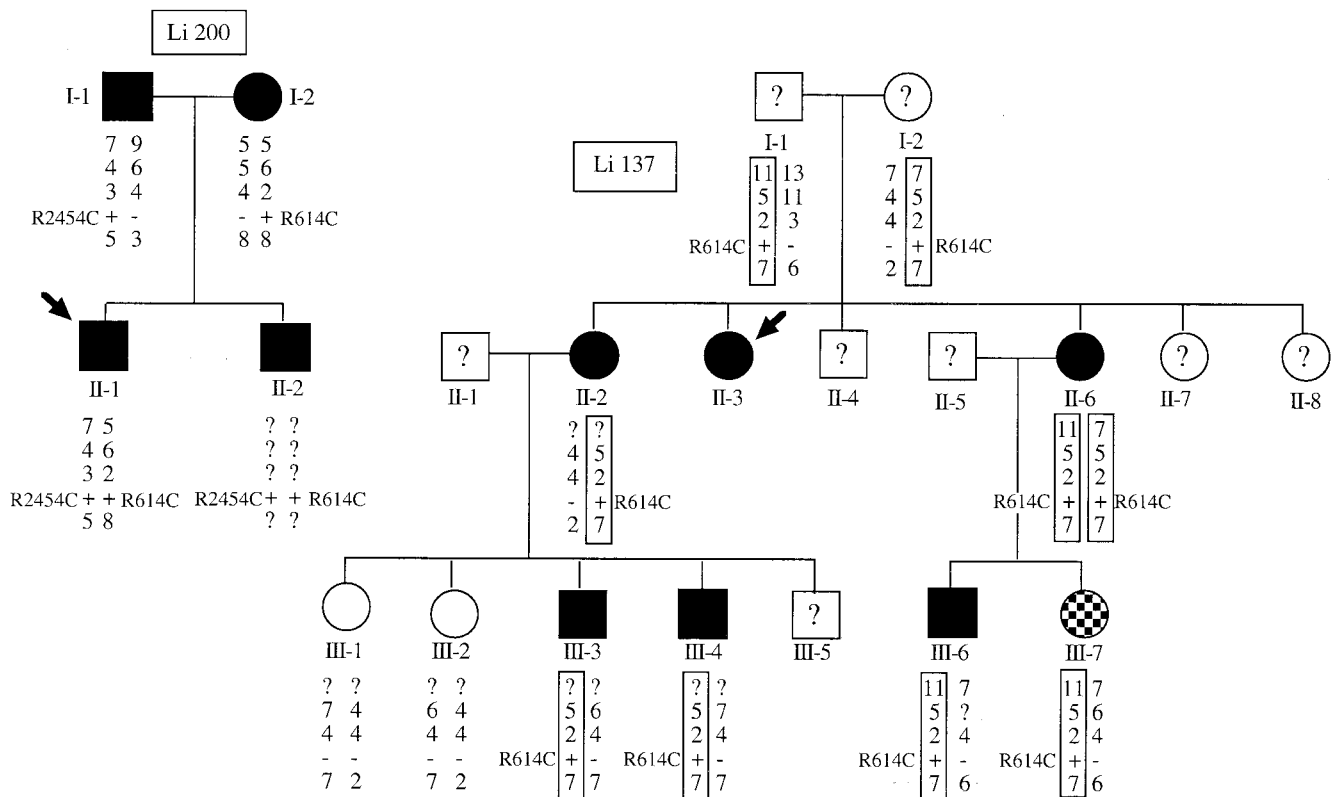
Molecular genetic studies have established the skeletal muscle ryanodine receptor (RYR1) on chromosome 19 as the primary MHS locus.<sup>7,8</sup> To date, at least 26 different mutations of the *RyR1* gene have been associated to the MHS phenotype.<sup>9-14</sup> Genetic heterogeneity has been reported in MH, and alternative MHS loci have been proposed on chromosomes 17q11.2-q24,<sup>15</sup> 7q11.23-q21.1,<sup>16</sup> 3q13.1,<sup>17</sup> and 5p.<sup>18</sup> However, no candidate genes associated with the disease have been identified in these regions. More recently, an MHS locus was identified on chromosome 1q, and a causative mutation was identified in the *CACNA1S* gene that encodes the  $\alpha_1$  subunit of the dihydropyridine receptor.<sup>19</sup>

Because diagnosis based on IVCT requires a muscle biopsy specimen, alternative diagnostic approaches, including nuclear magnetic resonance spectroscopy<sup>20,21</sup> and genetics, have been considered. Along this line, the European MH Group recently issued guidelines for the use of genetics as a diagnostic tool.<sup>22</sup> As the presence of

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Received from the Laboratoire de Biochimie de l'ADN-BECP/EA2943, Centre Hospitalier Universitaire CEA and Université Joseph Fourier, Grenoble, France. Submitted for publication September 27, 2001. Accepted for publication June 3, 2002. Supported in part by grants from the Association Française contre les Myopathies, Paris, France, Institut National de la Santé Et de la Recherche Médicale, Paris, France, Programme Hospitalier de Recherche Clinique du Centre Hospitalier Universitaire de Grenoble, Grenoble, France, and the Fondation Daniel Ducoin, Paris, France.

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**Fig. 1.** Pedigrees of families Li 200 and Li 137 showing the *in vitro* diagnostic test status, the constructed chromosome 19q13.2 haplotypes, and the presence of the RYR1 mutations. Arrows denote the proband; black symbols = malignant hyperthermia susceptible; white symbols = malignant hyperthermia normal; checkered symbols = malignant hyperthermia equivocal to halothane; ? = not tested. The results of marker typing and mutation identification are shown in the following order: D19S191–D19S220–RYR1–mutation–D19S422.

a second MHS gene segregating in the kinship of an MH family must always be considered, the guidelines still recommend IVCT investigation in the members of an MH family who do not carry the mutation present in the MHS individuals of the family. This hypothesis had been previously used to support the recommendation to use IVCT to test both parents of an MHS proband. We describe six nonconsanguineous families that harbor at least two different causative genetic traits associated with MHS.

## Materials and Methods

### Patient and Families

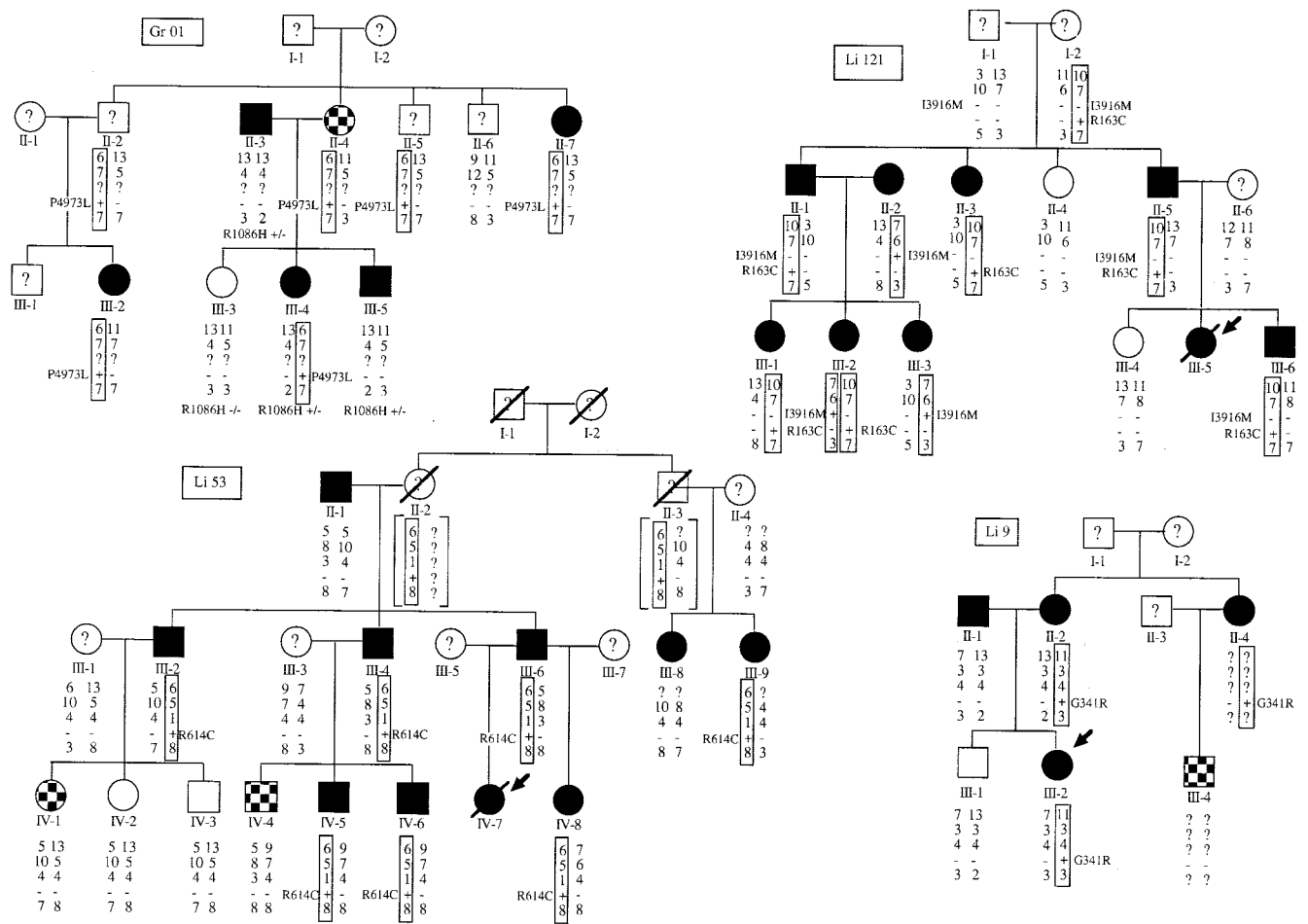
Data issued from a collaborative study that associated the Laboratory of Molecular Genetics from Grenoble and the French MH IVCT centers of Lille, Grenoble, Marseille, and Paris were available for 104 unrelated MH patients or MH families. In all cases, informed consent of the patients was obtained. In six nonconsanguineous families (Gr01, Li9, Li53, Li121, Li137, and Li200), both parents of an MHS patient were defined as MHS either on the basis of IVCT or the presence of a causative mutation. Pedigrees for documented families are shown in figures 1 and 2. Human studies presented received the

formal approval of the Comité Consultatif pour la Protection des Personnes en Recherche Biomédicale of the Center Hospitalier Universitaire de Grenoble (Grenoble, France).

At age 21 yr, patient II-1 of family Li 200 (fig. 1) underwent general anesthesia with isoflurane for shoulder surgery. After 45 min of anesthesia, he developed hyperthermia (38.2°C), tachycardia (120 beats/min), and hypercapnia (71 mmHg). He was later found to have a blood creatinine kinase (CK) concentration of 57,400 IU/l at day +1. He was successfully treated with dantrolene sodium (120 mg) and recovered without sequelae.

Proband of family Li 137 (patient II-3) was aged 30 yr and developed an MH crisis during general anesthesia with halothane (fig. 1). She was found to have a hyperthermia (41.5°C), hypercapnia (84 mmHg), and tachycardia. Twenty minutes after induction, she was treated with dantrolene sodium and recovered without sequelae.

The proband of family Li121, patient III-5 of the pedigree presented in figure 2, died from an MH crisis at age 13 yr during general anesthesia with halothane for reduction of ulna and radius fracture. She developed hyperthermia, ventricular arrhythmia, and generalized collapse. Dantrolene sodium was unsuccessfully administered 2 h after induction.



**Fig. 2.** Pedigrees of families Li 9, Li 53, Li 121, and Gr 01 showing the *in vitro* diagnostic test status, the constructed chromosome 19q13.2 haplotypes, and the presence of the RYR1 mutations. Arrows denotes the proband; black symbols = malignant hyperthermia susceptible; white symbols = malignant hyperthermia normal; checkered symbols = malignant hyperthermia equivocal to halothane; ? = not tested. The results of marker typing and mutation identification are shown in the following order: D19S191–D19S220–RYR1–mutation–D19S422, except for family Li121, where the order is D19S191–D19S220–mutation 1–mutation 2–D19S422. In family Gr 01, the presence of the R1086H of the CACNA1S gene is indicated below the chromosome 19q13.2 haplotype. Haplotypes of individuals II-2 and II-3 in family Li 53 have been reconstructed.

Patient III-2 of family Li 9 developed two episodes of postsurgery hyperthermia (41.5 and 42°C) after general anesthesia with halothane for otorhinolaryngology surgery.

At age 13 yr, patient IV-7 of family Li 53 underwent general anesthesia with halothane for surgery of ankle ligamenta. Ten minutes after induction, she developed muscle rigidity, hyperthermia (41.5°C), tachycardia, acidosis, and hypercapnia. She was later administered 120 mg of dantrolene sodium. She then developed asystolia and underwent cardiopulmonary reanimation. She died at day 14 from acute multivisceral failure.

**In Vitro Contracture Testing**

The IVCT of patients presented in this report were performed in the French MH centers of Lille and Grenoble. Muscle bundles were tested according to the caffeine-halothane contracture test protocol of the European Malignant Hyperthermia Group.<sup>6</sup> According to

this protocol, a patient is diagnosed as MHS if the 2-mN threshold value for muscle tension is attained at concentration of less than 2 mM caffeine and less than 2% halothane. A patient is classified as MHE to halothane (MHEh) if the 2-mN threshold value is attained only for a concentration of less than 2% halothane. When indicated, patients were also tested according to the North American protocol.<sup>5</sup>

**Haplotyping Analysis**

When possible, DNA from members of the different families was haplotyped as described previously using the following markers: D19S191, D19S220, RYR1, and D19S422.<sup>23</sup> When necessary, additional D19S190 and D19S223 markers were used. Genomic DNA was obtained either from the Genethon Bank (AFM, Evry, France) or extracted from whole blood using a rapid guanidine method.<sup>24</sup> The chromosome 19q13.2 haplo-

types of families GR01, Li9, Li53, Li121, Li 137, and Li200 are presented in figures 1 and 2.

#### *Malignant Hypertbermia Susceptibility Mutation Screening*

C35R, R163C, G248R, G341R, I403 M, Y522S, R552W, R614C/L, R2163C/H/P, V2168 M, T2206 M/R, G2434R, R2435H/L, R2454H/L, R2458C/H, and T4826I mutations previously described in MHS families were analyzed by restriction enzyme analysis or by single strand conformation analysis and sequencing. Numbering was done according to Zorzato's cDNA sequence<sup>25</sup> corrected by Phillips *et al.*<sup>26</sup> For restriction analysis, 5  $\mu$ l of amplified products was digested by 5 units of the appropriate enzyme for 3 h: C35R (*Aci* I), R163C (*Bst* UI), G248R (*Eco* NI), G341R (*Ssp* BI), R614C/L (*Bst* UI, *Rsa* I), R552W (*Mbo* I), and T2206 M/R (*BsmI* I). Digest products obtained following conditions recommended by the manufacturer were separated on 8 or 15% acrylamide gels depending on the size of the fragments. C35R, I403 M, Y522S, and R552W mutations present in exons 2, 12, 14, and 15, respectively, were analyzed by single strand conformation analysis and sequenced as previously described.<sup>27</sup> Mutations R2163C/H/P and V2168 M were analyzed by direct sequencing of exon 39. For patient II-7 of family GR01 and patient II-2 of family Li121, total RNA was extracted from a frozen muscle specimen, then first-strand cDNA synthesis and sequencing of the entire cDNA using overlapping fragments were performed as previously described.<sup>27,28</sup> The *Bst*-HKA I restriction site created by the I3916 M mutation was used for segregation analysis of the mutation in the Li121 family. The *Aci* I restriction site abolished by the P4973 I mutation was used for segregation analysis of the mutation in the GR01 family. One hundred chromosomes of the general population were then tested for these mutations by d-high-performance liquid chromatography using samples from mutated patients as standards.

## Results

#### *Family Li 200*

After full recovery without sequelae from his MH episode, patient II-1 was defined as MHS according to the European IVCT protocol. His two parents and his brother also tested positive for MHS (table 1). Mutation screening of the *RyR1* gene revealed the presence of the "pig" mutation, *i.e.*, the R614C mutation, in his mother's DNA (patient I-2) and of the R2454C mutation in his father's DNA, while proband II-1 and his brother (patient II-2) were compound heterozygous bearing both R614C and R2454C mutations (fig. 1).

#### *Family Li 137*

The IVCT investigations indicated that two sisters (patients II-2 and II-6) of the proband were MHS (fig. 1).

Both MHS sisters transmitted the MHS phenotype to their children (patients III-3, III-4, and III-6). From his muscle's response to halothane and caffeine, one of the children (patient III-7) was classified MHEh according to the European protocol. Molecular investigation of the family showed the presence of the R614C mutation in the *RyR1* gene. When investigated, both parents (individuals I-1 and I-2) proved to harbor the R614C mutation. As presented in figure 1, the two R614C mutations were associated to different haplotypes. However, while D19191 and D19S423 markers were different, these two individuals shared the D19S220, RYR1, and D19S422 polymorphisms located in or close to the *RyR1* gene. Therefore, one cannot formally exclude a distant common mutated ancestor for these two individuals. Patient II-6 was homozygous for the R614C mutation, and she transmitted the mutated allele to her two children. However, while her son (III-6) tested positive for MHS, her daughter, who had received the same R614C haplotype as her brother and that segregated with the MHS phenotype in the rest of the family, tested positive for MHEh.

#### *Family Li 121*

Pharmacologic and genetic investigations showed that the brother (III-6), the father (II-5), the aunt (II-3), the uncle (II-1), and two of the three cousins (III-1 and III-2) of the proband were MHS and shared the same R163C mutation of RYR1. A third cousin (patient III-3) who also tested positive for MHS did not bear the R163C mutation. When tested, her mother (patient II-2) was also classified as MHS. Histologic analysis revealed the presence of "core" pictures in muscle fibers of patient III-2; however, the patient did not present with clinical symptoms of myopathy, and the diagnosis of central core disease was clearly ruled out. Sequencing of the *RyR1* cDNA obtained from patient II-2 identified a unique 11748T>G mutation in exon 86 that leads to a change from a conserved isoleucine to a methionine at position 3916 of the RYR1 protein. The mutation was also identified in patients III-2 and III-3, who received the same chromosome 19q13.2 *RyR1* allele from their mother (II-2) and was not found in the rest of the family or in 100 chromosomes from the general population. Based on these data, one can reasonably speculate over the pathogenic role of this mutation. Therefore, patient III-2 received two different MHS genotypes associated with RYR1: the R163C mutation from her father and the I3916 M from her mother.

#### *Family Li 9*

The proband, her parents (patients II-1 and II-2), and her aunt tested positive for MHS using the European or the North American protocols. A G341R mutation in the *RyR1* gene was identified at a heterozygous level in the proband's DNA. As shown in figure 1, the mutation was also identified in her mother and aunt's DNA but not in

**Table 1. IVCT and CPK Data of MHS and MHE Individuals**

Families	Tension			CPK * (IU/l)	IVCT Status	Mutation	Age † (yr)
	Caffeine 2 mm (g)	Halothane 2% (g)	Halothane 3% (g) ‡				
Li 200							
I-1	0.40	4.40	3.90	243	MHS	RYR1:R2454C	51
I-2	0.20	0.70	2.10	81	MHS	RYR1:R164C	49
II-1	0.45	0.85	0.70	1102	MHS	RYR1:R2454C /R164C	21
II-2	1.35	1.45	1.30	1032	MHS	RYR1:R2454C /R164C	23
Li 121							
II-1	1.05	1.90	1.80	347	MHS	RYR1:R163C	48
II-2	0.90	2.90	1.60	84	MHS	RYR1:I3916M	47
II-3	1.90	3.00	6.00	107	MHS	RYR1: R163C	37
II-5	2.00	2.50	1.50	112	MHS	RYR1: R163C	41
III-1	1.45	2.00	5.40	165	MHS	RYR1:R163C	16
III-2	1.75	5.00	7.00	981	MHS	RYR1:R163C /I3916M	22
III-3	0.20	0.55	1.60	101	MHS	I3916M	23
III-6	1.20	1.40	2.80	63	MHS	RYR1:R163C	12
Li 137							
II-2	0.50	0.60	3.30	57	MHS	RYR1:R614C	39
II-6	1.65	1.00	6.10	331	MHS	RYR1:R614C/R614C	44
III-4	1.00	2.20	2.65	138	MHS	RYR1:R614C	18
III-5	0.45	2.15	3.10	144	MHS	RYR1: R614C	14
III-6	1.00	1.10	5.20	169	MHS	RYR1:R614C	26
III-7	0.05	0.55	0.40	106	MHEh	RYR1:R614C	13
Gr 01							
II-3	1.60	2.70	ND	ND	MHS	CACNL1A3:R1086H	63
II-4	0.10	0.20	ND	ND	MHEh	RYR1:P4973L	55
II-7	0.30	0.60	ND	87	MHS	RYR1: P4973L	49
III-2	0.40	0.80	ND	67	MHS	RYR1:P4973L	33
III-4	0.30	1.40	ND	272	MHS	CACNL1A3:R1086H RYR1:P4973L	32
III-5	0.20	0.40	ND	nd	MHS	CACNL1A3:R1086H	33
Li 9							
II-1	0.20	0.25	0.90	96	MHS	?	48
II-2	0.90	1.50	1.25	58	MHS	RYR1:G341R	51
II-4	0.25	2.95	3.35	47	MHS	RYR1:G341R	49
III-2	0.60	0.45	ND	92	MHS	RYR1:G341R	19
III-4	0.05	0.70	0.40	124	MHEh	?	20
Li53							
II-1	0.20	0.50	ND	34	MHS	?	—
III-2	0.25	0.50	ND	48	MHS	RYR1:R614C	—
III-4	0.30	0.60	ND	46	MHS	RYR1:R614C	—
III-6	0.27	0.20	ND	52	MHS	RYR1:R614C	—
III-8	0.50	1.50	ND	49	MHS	?	49
III-9	0.55	1.20	ND	97	MHS	RYR1:R614C	47
IV-4	0.00	0.20	ND	75	MHEh		—
IV-5	0.40	0.80	ND	94	MHS	RYR1:R614C	—
IV-6	0.20	0.30	ND	87	MHS	RYR1:R614C	—
IV-8	0.20	0.20	ND	56	MHS	RYR1:R614C	—

\* n < 120; normal value < 120. † age of the patient when biopsy was performed. ‡ protocol according to Larach.<sup>5</sup>

CPK = creatine phosphate kinase; IVCT = *in vitro* contracture testing; MHE = malignant hyperthermia equivocal; MHEh = malignant hyperthermia equivocal to halothane; MHS = malignant hyperthermia susceptibility.

her father’s DNA. Patient III-4, who did not receive the G341R mutation from her MHS mother, was classified as MHEh.

**Family Li 53**

Muscle testing indicated that a number of patients could be classified as MHS in the family (fig. 2). However, linkage analysis using chromosome 19q13.2 polymorphic markers apparently excluded the RYR1 locus from being associated with the disease in the family or

suggested a genetic heterogeneity (fig. 2). The hypothesis of the presence of more than one MHS trait in the pedigree was suggested by the identification of the “pig” mutation R614C mutation in all (IV-5, IV-6, IV-8, III-2, III-4, III-6, III-9) but two (II-1, III-8) of the MHS patients. IVCT data from these two MHS patients could not be distinguished from those of other MHS patients of the family (table 1). The genetic analysis of the pedigree indicated that the R614C mutation originated from one of the two grand-grand parents (patient I-1 or I-2). No

mutation has been yet identified in the two genetically unrelated MHS patients (II-1 and III-8).

#### Family Gr 01

Patient II-3 of the family presented in figure 2 belonged to a large MHS family in which an R1086H mutation responsible for the MHS trait was identified in the *CACNAIS* gene.<sup>19</sup> The ICVT data indicated that two of his children (patients III-4 and III-5) were MHS, while his wife (patient II-4) was classified as MHEh (table 1). As shown in figure 2, patient II-3 transmitted the R1086H mutation to his affected children. Additional IVCT investigations of patient II-4's family indicated that her sister (patient II-7) and her niece (patient III-2) were MHS. Linkage studies in this branch of the pedigree were compatible with the MHS trait being associated with the *RyR1* gene (boxed haplotype of the Gr01 family in fig. 2). Sequencing of the *RyR1* cDNA obtained from patient II-7 identified a 14918 C>T mutation in exon 104 that leads to a change from a conserved proline to a leucine at position 4973 of the RYR1 protein. The mutation was also identified in patients II-4, II-2, III-2, and III-4, who received the same *RyR1* allele, and was not found either in the rest of the family or in 100 chromosomes from the general population. Based on these data, one can reasonably argue over the pathogenic role of the L4793P mutation. Patient III-4 received two different MHS alleles: the R1086H mutation in the *CACNAIS* gene from his father and a second *RyR1* mutated allele from his mother: the P4973L mutation. This is the first report of a compound heterozygous patient with mutations associated with two different MHS loci.

As shown in table 1, the presence of a mutation in both alleles of the *RyR1* gene in either homozygous patient (individual II-6 of family Li137: R614C/R614C) or in compound heterozygous patients (individuals II-1 and II-2 of family Li200: R2454C/R614C or patient III-2 of family Li121) did not induce stronger contractile responses to caffeine or halothane. This situation was also observed for patient III-4 of family GR01, who is a compound heterozygous with mutations in two different genes.

Interestingly, in families Li121, Li 137, and Li 200, resting CK concentrations of homozygous patients (Li137: II-6) or compound heterozygous patients (Li121: III-2; Li200: II-1 and II-2) differed significantly from values measured in heterozygous MHS patients (fig. 3).

## Discussion

The six different pedigrees of MHS families presented here illustrate different situations showing the presence of more than one genetic trait associated with MHS in a family. In family Li137 (figure 1), patient II-6, who is homozygous for the R614C mutation, received a mu-

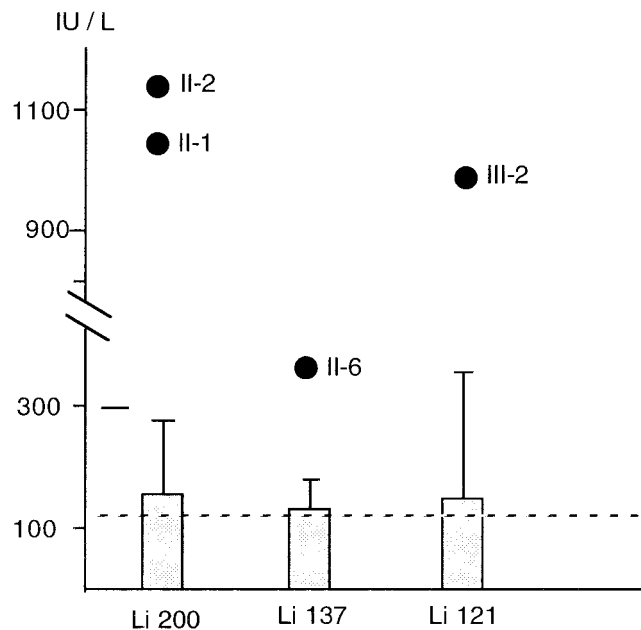


Fig. 3. Creatinine kinase (CK) analysis of homozygous or compound heterozygous patients. Bars represent the mean value  $\pm$  2 SD of the CK values determined in heterozygous malignant hyperthermia-susceptible (MHS) patients. Black dots represent CK values measured in homozygous MHS patients (Li 137/II-6) or compound heterozygous patients (Li 200/II-1, II-2; Li 121/III-2). The horizontal dotted line corresponds to the upper level for normal values of CK (N < 120 IU/L).

tated *RyR1* allele from both her mother (individual I-2) and her father (individual I-1). Homozygote patients for RYR1 mutations have been described previously in the literature.<sup>29,30</sup> Situations of Li200 and Li121 are more original since in these two families, two different mutations of the *RyR1* gene have been identified in the progeny of two unrelated individuals. This is the first report of compound heterozygote patients with two different RYR1 mutations: R614C/R2454C in patients II-1 and II-2 of family Li200, and I3916 M/R163C in patient III-2 of family Li121. The situation of family GR01 is even more original since in that family patient III-4 received the R1086H mutation of the *CACNAIS* gene from his father and the P4973 I mutation in the *RyR1* gene from her mother. This is the first report of a compound heterozygote bearing two different mutations on two different MHS causative genes.

In the two other families, only one trait has been characterized at the molecular level. In pedigree Li 53, while the presence of the R614C mutation responsible for MHS has been identified in some members of the family, two unrelated patients (II-1 and III-8) tested positive for MHS without the mutation (table 1). In family Li9, both parents of the proband tested positive for MHS; however, only the mother had the RYR1 mutation identified in her daughter. One possible explanation for these two situations is the occurrence of false-positive IVCT results in these three unrelated patients. Alterna-

tively, one can also consider the presence of a second genetic cause of MHS in these two families.

Occurrence of positive IVCT results in individuals affected by neuromyopathic syndromes have been reported<sup>31,32</sup>; however, none of the MHS patients included in this study presented with clinical symptoms. On the other hand, false-negative results with IVCT have been reported.<sup>33</sup> Therefore we critically checked the IVCT and genetic data of the 41 MHS families for which a mutation had been characterized in our 104 MHS families. None of the 95 MH normal patients was found to harbor a mutation in the *RyR1* or in the *CACNA1S* genes.

Of course, the occurrence of false-positive IVCT results in families Li53 and Li9 cannot be ruled out. However, considering the strong experience of the investigators, the use of well-standardized protocols, and the IVCT data, the presence of a second genetic allele responsible for MHS in these two families can be a plausible explanation. However, the absence of a frozen muscle specimen for the suspected patients did not allow a full screening for mutations in the *RyR1* gene.

The observation that some patients were homozygous or compound heterozygous raised the question of their clinical or biologic phenotype. As shown in table 1, homozygous or compound heterozygous patients could not be differentiated on the basis of their IVCT results. This is in agreement with other previous reports.<sup>30</sup> An elevation of the blood CK concentration had been previously described in MHS patients.<sup>31</sup> However, such elevation did not always correlate with the MHS status. This may be the consequence of the nature of the MHS mutations or of the specific familial genetic backgrounds. The CK serum concentration was investigated in several families. Interestingly, the CK concentrations were clearly elevated ( $> +2$  SD) in homozygous or compound heterozygous MHS patients (patients II-1 and II-2 in family Li 200, patient III-2 in family Li 121, and patient II-6 in family Li 137) when compared with CK concentrations found in the respective heterozygous MHS populations. It should be noted that muscle of patient III-2 of family Li 121 showed the presence of cores in fibers. One can, of course, speculate that the CK elevation or the histologic features are related to the cumulative effect of two different MHS traits in these specific families. However, a more extensive study including a larger number of patients and families is needed to draw more definitive conclusions about the physiologic significance of the CK concentration in homozygous or compound heterozygous MHS patients.

The MHEh status, e.g., patients with a positive response to halothane but a negative one to caffeine, is a phenotypic status generated by the European IVCT protocol. When we considered our total 41 MHS families harboring an identified mutation, 31 of the 264 tested patients were classified as MHEh, with 9 bearing an *RYR1* mutation. Direct diagnosis of MHS patients can be

based on the identification of a pathogenic mutation,<sup>22</sup> and these 9 MHEh patients would be defined as MHS from a genetic point of view. On the other hand, the 22 MHEh patients who did not have a mutation could not be considered as MH normal because the presence of another MHS allele cannot be ruled out in these patients. This is clearly illustrated by the situation of family Li 9, in which the presence of two different MHS traits is suspected. In this family, patient III-4 tested MHEh and did not have the G341R mutation identified in her mother. However, he is still clearly at risk to harbor a not-yet-identified MHS mutation.

The characterization of 4 families presenting with two independent genetic traits associated with MHS from a total of 104 French MHS families raised the question of the frequency of the genetic defect in the general population. Based on the analysis of crisis reports, an incidence of 1 in 10,000 is usually admitted for this autosomal dominant disease. Considering such figures, the probability to characterize nonconsanguineous homozygous or compound heterozygous MHS patients should be very low:  $1/10^4 \times 1/10^4 \times 1/4 = 1/4 \times 10^8$ . Therefore, considering the size of the French population ( $6 \times 10^7$  inhabitants), we should not have identified more than one such case. As MHS individuals do not usually present with specific clinical symptoms, one can rule out a possible bias due to interaction between MHS families through patients' association meetings, for example. Furthermore, both parents have not been tested in all families, and not all French MHS families have been characterized at a molecular level; therefore, the 4 families with two independent MHS traits is a conservative value. Therefore, taking in account the number of unrelated homozygous or compound heterozygous individuals for the MHS trait characterized in this study, an autosomal dominant mode of inheritance of the disease, and the size of the French population, the 1 in 10,000 value taken for MHS incidence in the general population is likely to be underestimated. While only an extensive screening of thousands of chromosomes will bring a precise figure, one can roughly estimate from our combined IVCT and genetic data that a value of 1 in 2-3,000 for the incidence of the MHS trait in the French population would be closer to the truth. The apparent discrepancy between this and previous evaluations of the disease frequency based on anesthetic crisis may be explained by a lower than usually considered (98%) penetrance value for the disease.

The documented presence of more than one genetic trait in a significant number of MH families is also important when considering the genetic heterogeneity of MH. While six MHS loci have been described so far, mutations have been identified in only two genes, *RyR1* and *CACNA1S*, with the latest one involved only in a few families.<sup>19,34</sup> Characterization of genetic heterogeneity usually results from linkage studies that aim at the asso-

ciation of a specific haplotype with the disease in a given family. Using such an approach, and in absence of both parents having been tested, the RYR1 locus on chromosome 19q13.2 would have been excluded on the basis of linkage analysis in some families described here. Another situation that may lead to erroneous conclusions regarding a possible genetic heterogeneity in a family is the occurrence of neomutations in the *RyR1* gene.<sup>19,28</sup> From all of these considerations, it appears that conclusions of genetic heterogeneity must take in account (1) a critical evaluation of the IVCT data used to define the MH status of patients, (2) the combined use of IVCT and DNA analysis whenever possible in each MH family, (3) testing both parents of MHS probands to rule out the presence of a second genetic origin of the disease, and (4) the hypothesis of a neomutation of a gene causing MHS.

The authors thank all family members for their participation in this study and Joëlle Lerault (Technician, Centre Hospitalier Universitaire de Grenoble, France) for technical support.

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