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Masseter Muscle Rigidity Associated with Glycine^{1306}-to-Alanine Mutation in the Adult Muscle Sodium Channel α-Subunit Gene

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Background: **Succinylcholine-induced masseter muscle rigidity (MMR) is a potentially life-threatening complication of anesthesia and is closely correlated with the heterogeneous disorder malignant hyperthermia (MH) susceptibility. MMR also is identified with a variety of neuromuscular disorders, including the myotonias, that are associated with abnormal *in vitro* contracture test (IVCT) results. Recently, mutations in the adult skeletal muscle sodium channel α-subunit gene (SCN4A) have been shown to cause generalized nondystrophic myotonias, some of which are associated with mild nonspecific symptoms. The purpose of the current investigation was to begin to evaluate the molecular genetic relationship between known mutations in the SCN4A gene, MMR, and the results of the IVCT used to diagnose MH-susceptibility.**

Methods: **A single extended pedigree of 16 individuals was ascertained through a proband who experienced MMR and whole-body rigidity after succinylcholine administration. Subsequently, four individuals were shown to have a mild form of myotonia on clinical and laboratory examination. IVCT was carried out according to standardized protocols. Mutations in the SCN4A gene were sought in exons 22 and 24 using single-strand conformational analyses. Variability in the SCN4A gene sequence was confirmed by direct DNA sequence analyses.**

Results: **Four individuals with myotonia were shown to carry a guanine-to-cytosine mutation at nucleotide position 3917 of the reported SCN4A sequence. This DNA mutation was coinherited with MMR and an abnormal IVCT result in this family. Previous studies have demonstrated that the glycine^{1306}-to-alanine substitution is associated with a mild clinical syndrome referred to as myotonia fluctuans.**

Conclusions: **The current report provides direct evidence that succinylcholine-induced MMR, whole-body rigidity, and an abnormal IVCT result are associated with a mutation in the SCN4A gene. (Key words: Malignant hyperthermia: *in vitro* contraction test; masseter rigidity. Molecular biology: genetic heterogeneity; mutations; sodium channel.)**

SUCCINYLCHOLINE-INDUCED masseter muscle rigidity (MMR) may prevent tracheal intubation, complicating airway management during anesthesia. MMR is closely correlated with malignant hyperthermia (MH) susceptibility as diagnosed by the *in vitro* contracture test (IVCT).[1] Currently, controversy exists regarding a

definition of MMR and the appropriate anesthetic management after MMR occurs.[1] Because this clinical finding is nonspecific, and the anesthetic is aborted in most cases with the earliest sign of MH-susceptibility, often, little if any laboratory data are available to confirm a diagnosis. Because of the strong association between MMR and MH-susceptibility, in the absence of diagnostic testing, the patient may be mislabelled as MH-susceptible. The diagnosis of MH-susceptibility may bring significant hardships to the patient and their entire family.[2] However, in most cases when the diagnosis is established, it is based solely on the results of the IVCT.

Succinylcholine-induced muscle rigidity, including MMR, is associated with other neuromuscular disorders.[3,4] Moreover, some of these myopathic conditions are associated with abnormal IVCT results.[3,5] One of these neuromuscular disorders, central core disease, appears to be allelic with, or caused by, mutations in the same gene as that associated with certain forms of MH-susceptibility.[6,7] However, an abnormal IVCT result in patients with myotonic dystrophy appears unlikely to be associated with MH-susceptibility.[8] For other neuromuscular disorders, such as the nondystrophic myotonias and the Schwartz-Jampal syndrome, the significance of an abnormal IVCT result or the association with MH-susceptibility is less clear.[3,8,9] Thus, continued molecular genetic studies will be necessary to develop a complete understanding of the association between MMR, an abnormal IVCT result, and MH-susceptibility.

The purpose of the current investigation was to begin to evaluate the molecular relationship between MMR, known mutations in a gene candidate, and the results of the IVCT used to diagnose MH-susceptibility. Recently, mutations have been described in the adult skeletal muscle sodium channel α-subunit gene (SCN4A) that are associated with various forms of generalized nondystrophic myotonia and hyperkalemic periodic paralysis.[10] Because some of these sodium channel myopathies are associated with mild nonspecific signs and symptoms,[11,12] they may not be recognized during the routine preoperative examination. We reasoned that mutations in SCN4A may explain some forms of MMR and MH-like episodes associated with an abnormal IVCT result.[13–15] We tested the hypothesis that specific mutations in the SCN4A gene are associated with MMR in patients referred for a diagnosis of suspected MH-susceptibility. Our data confirm that a mutation in this gene candidate is associated with succinylcholine-induced MMR, whole-body rigidity, and an abnormal IVCT result.

Materials and Methods

Phenotypic Evaluation

Established protocols were used as a standardized test to determine the MH-susceptibility phenotype and are based on skeletal muscle contractures *in vitro* to halothane or caffeine (IVCT).[16–19] The outcome of these established protocols provides the following diagnoses: MH-susceptible, MH-negative, or MH-equivocal.

We examined 16 individuals from one extended pedigree in detail. This family was ascertained through a proband who suffered a suspected episode of MH. The proband and her sister from US family five have been described previously.[3] Briefly, these individuals presented with masseter spasm and whole-body rigidity on induction of anesthesia after the administration of succinylcholine. The proband was then noted to be hypotensive (80/50), and she recovered uneventfully once the anesthetic was discontinued. Afterward, a family history was obtained of stiffness and muscle spasm with exercise, characterized by daily fluctuations in severity. Electromyograms and IVCTs were diagnostic for a form of myotonia and MH-susceptibility, respectively.

Isolation of Human Genomic DNA

Human genomic DNA was isolated and handled according to methods previously described.[14] Briefly, nuclei from whole blood samples were isolated and lysed, then DNA was purified by chloroform extraction and ethanol precipitation.

Conditions for the Polymerase Chain Reaction

Oligonucleotide primers were end-labelled with biotin during synthesis for solid phase sequence analyses or [^{32}P] γ-ATP (6,000 Ci/mmole) by polynucleotide kinase for single-strand conformational polymorphism analyses (SSCP), see below. Conditions for polymerase chain reaction (PCR) were: 0.4 μM primers, 1.5 mM $MgCl_2$, 50 mM KCl (final concentrations), and 0.5 U Taq polymerase; 94°C for 10 min; followed immediately by 30 cycles of 94°C for 30 s, and the appropriate annealing temperature for each primer pair of 30 s, and 72°C for 30 s; followed by 72°C for 7 min. To examine for mutations between codons 1305

to 1339, we selected the following primer sequences from exon 22: forward, TGGAGGCAGGAAGGGGAACT, and reverse, GGCAGCACACACAGGACAGG. To examine for mutations between codons 1430 and 1503, we selected the following primer sequences from exon 24: forward, GCATCTGCTTCTTCTGCAGC, and reverse, CTCGCTGCTCTCCTCTGTGG, and between codons 1581 and 1602, we selected the primers forward, AGCGTCCTCACTAGCTTCTC, and reverse, ATGCCCGACTCCTTCTTGAC.

Examination for DNA Polymorphisms Using SSCP

PCR primers were synthesized from the DNA sequence of the SCN4A gene.[20] Primers were selected from unique sequences surrounding each exon using OLIGO 4.0. SSCP was carried out largely as described elsewhere,[21,22] with modifications.[23] SSCP was conducted at room temperature with and without 10% glycerol, using 6% polyacrylamide gels at a cross-linking monomer concentration of 2.67%. Twelve and one half microliters of PCR product was mixed with 12.5 μl 2× stop buffer (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol), denatured at 85–90°C for 8 min, and immediately placed on ice. Electrophoresis was carried out at 12 W for approximately 11 h for gels containing glycerol and 5 h for gels without glycerol. Gels then were dried and exposed to either Kodak BIOMAX or XAR film.

Direct DNA Sequencing of PCR Products

The size of the PCR products was verified by comparison with standards of known length. The PCR products were sequenced directly using solid-phase techniques.[24] PCR products (20 μl) were incubated with 40 μl of Dynabeads m-280 (Dynal) for 10 min. The beads containing the bound products were then washed as follows: binding and wash (B & W) buffer containing 10 mM tris-HCl *p*H 7.5, 1 mM EDTA, 2 M NaCl, denatured with 0.1 N NaOH, and then with 0.1 N NaOH, B&W buffer, and finally with TE (10 mM Tris-HCl *p*H 8 and 1 mM EDTA), as suggested by the manufacturer. The pellet of beads was resuspended with 10 μl of sterile double-distilled water. Sequencing reactions were carried out using Sequenase 2.0 (USB) and dideoxy termination methods.[25] Unlabelled oligonucleotides for exon 22 served as the sequencing primers. [^{35}S] or [^{33}P] α-dATP was incorporated into the sequencing reaction, and the products were electrophoresed through 6% polyacrylamide gels containing 7 M urea. Gels were dried without fixing and exposed to x-ray film. Genotypes were scored for each family member by noting variable bands in the DNA fragment ladder. Alleles were determined by comparing the genotypes of parents and offspring.

Mutation Detection by Restriction Fragment Length Polymorphism Analyses

PCR products were obtained as above and incubated at 37°C for 60 min in the presence of 5 U of Aci I enzyme and 1× buffer #3 as supplied by New England Biolabs, Inc. This mixture underwent electrophoresis on 2% agarose gels with standards of known size and stained with ethidium bromide.

Results

A single DNA polymorphism was identified in exon 22 using SSCP from this nuclear family (fig. 1). This polymorphism occurred only in individuals diagnosed with myotonia and MH-susceptibility or in those affected with myotonia but who were not tested by IVCT. Direct DNA sequencing of this exon revealed a single base substitution at nucleotide position 3917 in these individuals (fig. 2). As a result of this polymorphism, an alanine is substituted for a glycine at amino acid position 1306 within the cytoplasmic loop connecting repeats III and IV of this adult skeletal muscle sodium channel subunit.

IVCT results are available on the three individuals II-2, II-3, and II-6 from US family five (fig. 2). Individual II-6 was diagnosed as MH-negative on at least three separate muscle strips by IVCT with both halothane and caffeine and was unaffected with myotonia by history and examination. Individuals II-2 and II-3 are presumed MH-susceptible with diagnostic contractures at 3% halothane of 1.9 and 1.3 g, respectively. Contractures elicited at 2 mM caffeine in three separate muscle strips from II-6, II-3, and II-2 were within normal limits. History and/or clinical examination of individuals I-1, II-4, II-3, and II-2 revealed evidence of myotonia. II-1, II-6, and III-2 displayed no evidence of myotonia by history or neurologic examination. Electromyogram data confirm these diagnoses for individuals II-2, II-3, II-6, and III-2.

We developed a simple rapid noninvasive test to detect the glycine^{1306}-to-alanine mutation using a PCR-based assay. A restriction fragment length polymorphism is produced by this guanine-to-cytosine mutation at nucleotide

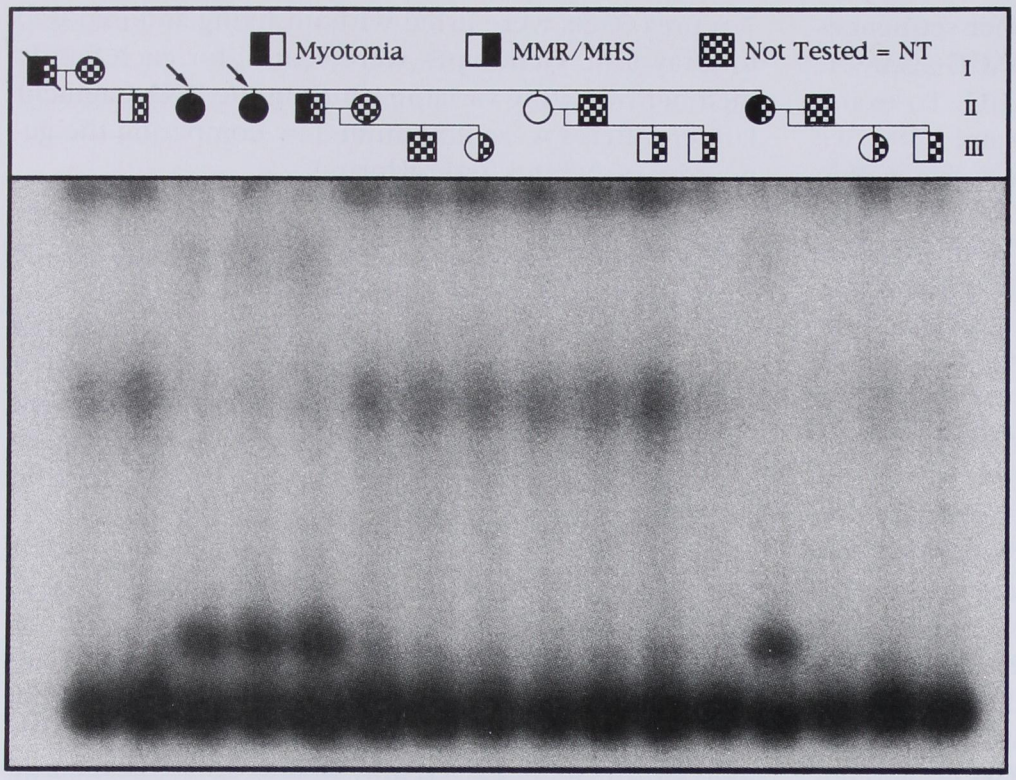

Fig. 1. Extended pedigree showing relationship between myotonia or malignant hyperthermia susceptibility/masseter muscle rigidity and the single-strand conformational polymorphism analyses results on genomic DNA amplified by polymerase chain reaction from exon 22 of the sodium channel α-subunit gene gene. An abnormal migrating band in these individuals is indicative of the glycine^{1306}-to-alanine substitution.

3917 of the SCN4A gene. This base change produces the addition of an Aci I restriction enzyme site. When the 199 base PCR product is digested with Aci I in affected individuals, it is cleaved into two fragments of 139 and 60 bases in length, which are easily detected by gel electrophoresis (data not shown). This assay is diagnostic for this particular mutation.

Discussion

MMR is closely associated with MH-susceptibility and a number of myopathic conditions, including central core disease, Becker's dystrophy, arthrogryposis, and the myotonias.[1–5] It is now well recognized that MMR is likely to be a heterogeneous disorder and that certain forms do not progress to a recognizable hypermetabolic state.[26] Frequently, patients with myopathic disorders identified with MMR have an abnormal IVCT result, which is used to diagnose MH-susceptibility.[3–5,27] The anesthetic is often aborted when MMR occurs, assuming it is the first evidence of MH-susceptibility. Because MMR is a nonspecific sign, there is usually little clinical evidence to distinguish which patients would have proceeded to a hypermetabolic response characteristic of MH-susceptibility. In most cases, these individuals are either labelled as MH-susceptible without confirmation, or a diagnosis is sought later by the IVCT. Although the molecular basis for an abnormal IVCT result is also not known, we and others have shown that the genetic cause of an abnormal IVCT result is likely to vary among families diagnosed with MH-susceptibility.[14,15,28–30] This genetic heterogeneity is still largely unexplained, as is the association of an abnormal IVCT result with MMR and most of these other myopathic disorders. However, it suggests that a number of molecular defects can have as a common endpoint the elevation of myoplasmic calcium, resulting in sustained contractions or contractures, as well as an abnormal IVCT result.

Generalized nondystrophic myotonia is characterized by uncontrollable muscle stiffness due to membrane hyperexcitability. Myotonia is common to a group of disorders that vary significantly in severity and can be caused by either sodium or chloride channel dysfunction. The adult skeletal muscle sodium channel α-subunit gene designated SCN4A recently was localized to human chromosome 17q.[31] Numerous mutations in this gene have been identified that cause hyperkalemic periodic paralysis and various forms of generalized myotonia.[10] In the current report, we demonstrate that a mutation in the SCN4A gene is associated with succinylcholine-induced MMR and whole-body rigidity. Together, these findings generally are accepted as evidence that the likelihood of MH-susceptibility is

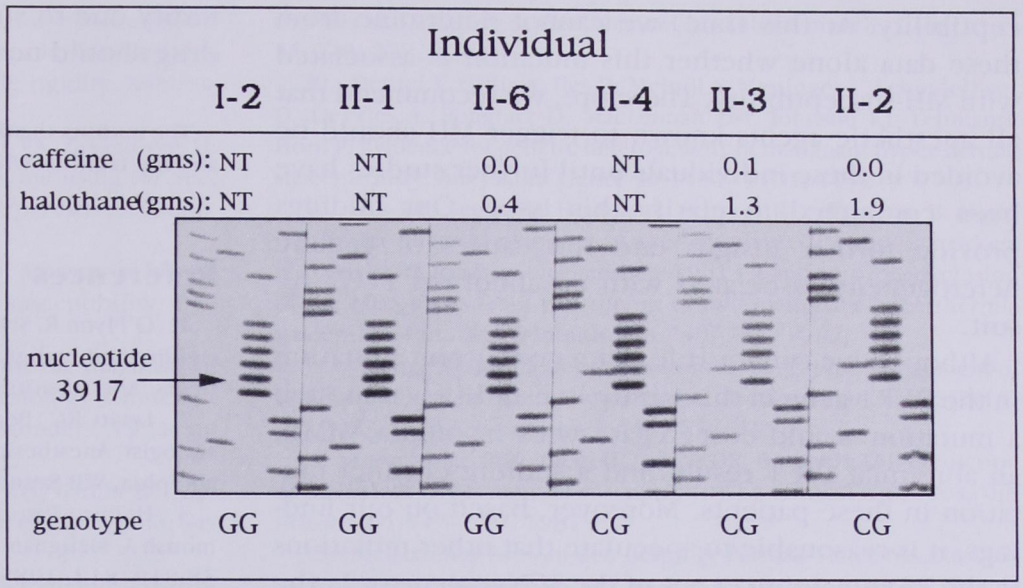

Fig. 2. Nuclear family showing relationship between malignant hyperthermia (MH) susceptibility/masseter muscle rigidity (MMR), myotonia, the *in vitro* contracture test result, and a segment of the DNA sequence from exon 22 of the sodium channel α-subunit gene gene. Note the guanine-to-cytosine mutations at position 3917 in exon 22 of the adult skeletal muscle sodium channel α-subunit gene in individuals II-2, II-3, and II-4. An alanine is substituted for a glycine at position 1306 as a result of this mutation. The arrow indicates individuals who experienced MMR and were referred for evaluation because of a suspected MH-like reaction to anesthesia. NT = not tested.

great.[32] However, these clinical signs by themselves are nonspecific, and these patients did not demonstrate hypermetabolism despite an abnormal IVCT result diagnostic of MH-susceptibility. Moreover, because this mild form of myotonia is difficult to identify on routine preoperative examination, and many patients refuse additional invasive testing after MMR is observed, it is unclear how many of these individuals are labelled as MH-susceptible.

The glycine at position 1306 is invariant across species, suggesting it is critical in the function of the α-subunit protein.[11] In addition, amino acid substitutions at this site are associated with electrophysiologic evidence of slowed inactivation of the sodium channel.[11] Altered sodium channel inactivation can produce muscle stiffness because of repeated or sustained action potentials, which result in prolonged contractions, contractures, or difficulty in relaxation. Although this mutation appears to be causal in myotonia, no information is available on what effect it has on muscle function in the presence of anesthetics. In particular, it remains unclear whether myoplasmic calcium regulation is significantly altered at rest and/or after exposure to triggering anesthetics. The substitution of an alanine for a glycine at position 1306 (mutation at nucleotide 3917) is associated with a distinctly abnormal IVCT result in each of the two individuals we tested. In contrast, a methionine substitution at amino acid 1313, associated with a more severe form of generalized myotonia termed paramyotonia[33] is not consistently associated with an abnormal IVCT result.‡‡ Lehmann-Horn and Iaizzo have suggested that an abnormal IVCT result does not necessarily imply that a myotonic individual has MH-susceptibility.[8] However, in their study, only individuals with myotonic dystrophy demonstrated a clearly abnormal IVCT result, and patients with generalized nondystrophic forms of myotonia produced normal or equivocal IVCT results.[8] In contrast, the IVCT from individuals II-2 and II-3 from family 5 were clearly abnormal. Thus, the relationship between this specific mutation and MH-susceptibility remains unclear.

Our patients recovered uneventfully from exposure to succinylcholine and anesthetics that trigger MH-susceptibility. However, based on the IVCT results, we cannot exclude the possibility that this mutation is a rare cause of MH-susceptibility. Indeed, another myotonic disorder, the Schwartz-Jampal syndrome, is associated with electrophysiologic evidence of sodium channel dysfunction and altered regulation of myoplasmic free calcium.[34] These patients have reportedly developed hyperthermia during anesthesia.[9] The relationship between MH-susceptibility and these myotonias may become clearer after additional analyses. However, based on the available data, it may be appropriate to examine the sodium channel for a mutation(s) in patients with Schwartz-Jampal syndrome. Likewise, electrophysiologic examination of skeletal muscle from patients with SCN4A mutations at position 1306 would be useful to understand whether such a mutation is associated with altered regulation of myoplasmic calcium after exposure to anesthetics that trigger MH-sus-

‡‡ Levitt RC: Unpublished observations. 1994.

ceptibility. At this time, we cannot determine from these data alone whether this mutation is associated with MH-susceptibility. Therefore, we recommend that all anesthetic agents known to trigger MH should be avoided in these individuals until further studies have been conducted to clarify this issue. Our findings provide further insight into the causes of genetic heterogeneity associated with an abnormal IVCT result.

Although we cannot rule out a concurrent mutation in the RYR1 gene in this family, it is unlikely that such a mutation would cosegregate with myotonia, MMR, an abnormal IVCT result, and a sodium channel mutation in these patients. Moreover, based on our findings, it is reasonable to speculate that other mutations in the 23 remaining exons of the SCN4A gene or in the β-subunit gene of the sodium channel mapped to chromosome 19q13.1-q13.2[35,36] may produce succinylcholine-induced MMR, whole-body rigidity, and an abnormal IVCT result. Recently, mutations have been reported in the chloride channel on chromosome 7q that are associated with generalized nondystrophic myotonias.[37,38] It remains to be shown whether selected mutations in this gene are associated with MMR, whole-body rigidity, and an abnormal IVCT result.

The individuals in US family five have a mild sodium channel myopathy referred to as myotonia fluctuans.[11,12] Molecular genetic and electrophysiologic studies suggest that this mutation causes a form of nonprogressive nondystrophic myotonia.[12] Patients with this disorder often are unaware of an underlying myopathy, as were our patients. The glycine[1306]-to-alanine substitution is associated with very mild nonspecific signs and symptoms of stiffness and muscle cramping with exercise that fluctuate in severity.[11] In each case, our patient's clinical history and examination was consistent with this recently described syndrome, leading us to examine exon 22 of the SCN4A gene. This dominantly inherited disorder is associated with subtle clinical signs and symptoms. As in our patients, unless a careful family history and detailed neurologic examination is taken, the significance of these subtle findings may not be appreciated preoperatively. If results from the preoperative examination are ambiguous, electromyogram and molecular genetic testing, as demonstrated in this report, can aid the clinical diagnosis. The restriction fragment length polymorphism assay described herein can be used as a simple noninvasive diagnostic test for this sodium channel mutation. These individuals are at high risk for anesthetic morbidity due to succinylcholine-induced MMR, and this drug should not be administered.

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