A Strategy for DNA Sequencing of CASQ1 reveals no mutations associated with malignant hyperthermia

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Abstract

The aim of this study is to elucidate the relationships between susceptibility to MH (MHS) and genes encoding skeletal muscle Ca2+ channels and their accessory proteins. Selected exons of Calsequestrin1 demonstrated that no mutations or any novel genetic variants were identified in the UK malignant hyperthermia population. Investigating other candidate genes that contribute to the calcium release unit, e.g. HRC, different subunits of DHPR, and Calmodulin, may lead to the identification and characterisation of novel mutations or to the detection of association with MH susceptibility. Further linkage studies and DNA sequencing of other putative candidate genes other than RYR1, CACNA1S and CASQ1, are suggested to further elucidate MH phenotype/genotype discordance and MH heterogeneity.

Keywords: *Malignant hyperthermia (MH), DNA Sequencing of CASQ1*

1. Introduction

1.1   Malignant hyperthermia (MH)

Malignant Hyperthermia (MH) is a potentially life-threatening pharmacogenetic syndrome of skeletal muscle which was first described by Denborough and Lovell in 1960 (Denborough and Lovell, 1960). The condition is referred to as Malignant Hyperthermia or Hyperpyrexia due to the very high body temperature accompanying an MH episode (Cullen, 1966). The MH phenotype is a skeletal muscle disorder found in individuals with a genetic predisposition who

respond adversely to the administration of potent inhalational volatile anaesthetics and depolarizing skeletal muscle relaxants such as halothane and succinylcholine respectively. This adverse reaction is mainly manifested in increased body temperature and muscle rigidity (Ellis *et al.*, 1990).

Despite increasing awareness of the condition among anaesthetists, MH remains one of the uncommon causes of serious morbidity and death in otherwise healthy individuals undergoing surgery under general anaesthesia (Hopkins, 2000), Therefore, proper medical intervention and management of an MH episode is a critical issue.

1.1.1   Laboratory diagnosis of MH phenotype

MH is diagnosed in the laboratory by the *in vitro* contracture test (IVCT) in Europe using European Malignant Hyperthermia Group (EMHG) standard (1984), the caffeine halothane contracture test (CHCT) in North America (Larach and Group, 1989) or the skinned fibre test in Japan (Rosenberg *et al.*, 2002). The majority of laboratories use the EMHG developed IVCT test which is based on the hypersensitivity of contracture of fresh muscle biopsy, to caffeine or halothane.

To date, the IVCT test is the only method used for the diagnosis of MH and there is no simple, non-invasive test for MH, and IVCT is the gold standard of MHS diagnosis (Mathews and Moore, 2004). As IVCT is invasive, costly, time-consuming and performed only in specialized centres, researchers are seeking to develop a sensitive, convenient and less invasive test for the diagnosis of MH (Allen and Lopez, 2002). One possibility is genetic testing, which is less invasive, cheaper and has lower morbidity than IVCT, as it requires only a small sample of blood rather than a muscle biopsy (Anderson-Pompa *et al.*, 2008).

1.1.2   Excitation-contraction coupling

Excitation-contraction (EC) coupling is a physiological regulatory process which occurs within muscle cells to convert the electrical action potential stimulation in the muscle cell to initiate its contraction (Wingertzahn and Ochs, 1998). This mechanism is initiated by the activation of the type 1 ryanodine receptor (RyR1) calcium channel when dihydropyridine receptors (DHPR) are activated by depolarization in the T-tubule. This depolarisation causes conformational changes in the α_{1S} subunit of DHPR leading to opening of the RyR1 channel (Franzini-Armstrong and Protasi, 1997). This leads to calcium release from the SR to the cytoplasm. Subsequently, increasing calcium concentration in the cytoplasm enhances the attachment of calcium ions to contractile proteins, thus initiating muscle contraction.

1.1.2.1 Ryanodine receptors (RyR) calcium channel

The ryanodine RyR1 channel was named due to its high affinity binding to the plant alkaloid, ryanodine, which was utilized in pesticides to cause paralysis of skeletal insect muscle (Pessah *et al.*, 1985). Mutations in *RYR1* have been found to be associated with muscle disorders including malignant hyperthermia (MacLennan *et al.*, 1990), central core disease, multiminicore disease, exertional rhabdomyolysis (Capacchione *et al.*, 2010), and atypical periodic paralyses.

1.1.2.2 Dihydropyridine receptor channel (DHPR)

The dihydropyridine receptor (DHPR) calcium channel is also referred to as the L-type Ca^{2+} channel voltage-gated Ca^{2+} channel (VGCC) or Cav1.1, and it has a 430 kDa molecular mass (Takahashi *et al.*, 1987). The DHPR plays a dual role as a calcium channel and as a voltage sensor that activates RyR1 to release calcium (Posterino and Lamb, 2003).

1.1.2.3 Calsequestrin (CASQ)

There are two isoforms of CASQ, type 1 (CASQ1) expressed in skeletal muscle and type 2 (CASQ2) expressed in cardiac muscle and encoded by *CASQ1* and *CASQ2* genes, respectively (Sanchez *et al.*, 2011). Calsequestrin1 (CASQ1) is the major Ca^{2+} storage protein in SR in skeletal muscle and has a molecular mass of 44 kDa. It is localized to the interior of the SR membrane and is referred to as Calsequestrin because the protein sequesters Ca^{2+} ions in the SR (MacLennan and Wong, 1971). In addition, CASQ1 has a major role in calcium homeostasis and in buffering Ca^{2+} ions (Beard *et al.*, 2004). Calsequestrin represents approximately 27% of the junctional proteins and is considered the primary protein in calcium storage. The effect of calsequestrin on the RyR1 channel depends on its interaction with triadin and junctin, which are anchoring proteins. CASQ1 interacts with RyR1 and with the luminal domain of triadin in a Ca^{2+}-dependent manner (Guo and Campbell, 1995). CASQ1 regulates RyR1 activity according to luminal Ca^{2+} concentrations (Beard *et al.*, 2004). CASQ1 binding with Ca^{2+} results in a conformational change in CASQ1 (Ikemoto *et al.*, 1972).

In addition to its RyR1 interaction, CASQ1 interacts with Junctional proteins; junctin, triadin and JP-45, suggesting a molecular complex to regulate RyR1 activity. However, the complete functional characterisation of interactions between proteins in this complex has not been yet elucidated (Beard *et al.*, 2009).

Mutations associated with abnormal sarcoplasmic reticulum Ca^{2+} release in the sarcoplasmic reticulum of cardiac Calsequestrin2 were detected in *CASQ2* and associated with various cardiac defects (Viatchenko-Karpinski *et al.*, 2007; De la Fuente *et al.*, 2008; Kirchhefer *et al.*, 2010). Experimentally, CASQ2-null myocytes demonstrate increased SR Ca^{2+} and susceptibility to catecholaminergic ventricular arrhythmias (Knollmann *et al.*, 2006). In addition, *CASQ1*-KO mouse model investigations demonstrate abnormal excitation contraction coupling, MHS like symptoms, and anaesthetic and heat-induced sudden death, suggesting skeletal *CASQ1* may be a candidate gene for MH (Protasi *et al.*, 2009).

1.1.3   Molecular genetics of MH

Genetic testing for MH is the best potential alternative to the IVCT, due to its overall advantages including accuracy, convenience, and reduced invasiveness. Establishment of genetic testing of MH could prevent the development of an MH episode during anaesthesia if routinely used before surgery. A DNA diagnostic test was suggested in 1990, when a mutation in the *RYR1* gene was identified (McCarthy *et al.*, 1990). Since then, the sensitivity of the genetic test has been improved significantly as more mutations in *RYR1* have been added to the screening protocol

(Anderson-Pompa *et al.*, 2008). To date, there are 30 causative mutations in the *RYR1* that have been confirmed experimentally and characterised to be associated with the MH phenotype.

Despite advances in identification of RYR1 mutations and increased numbers of MH-diagnostic mutations with improving sequencing techniques, RYR1 mutations have been excluded as genetic causative defects in about 30% of UK MHS families (Robinson *et al.*, 2006). Although DNA sequencing of the entire *RYR1* gene has increased the detection rate of causative mutations, in other countries it has also been observed that RYR1 mutations do not account for all MH , e.g. only up to 60% of French MHS families (Monnier *et al.*, 2005), 64% of Swiss families (Levano *et al.*, 2009) and 70% of North American families (Sambuughin *et al.*, 2005) show RYR1 mutations.

MH susceptibility has been linked genetically to the chromosomal locus 1q32 of the *CACNA1S* gene in a French MHS family (Monnier *et al.*, 1997). A pathological mutation, p.Arg1086His, in exon 26 of this gene was identified and documented as being associated with MHS in pedigrees (Monnier *et al.*, 1997). However, this mutation represents only 1% of MHS individuals (Sl Stewart, 2001).

1.1.4   Other putative candidate genes

Several potential candidate genes may contribute to the MH pathophysiology and susceptibility other than the major mutated protein RyR1 and alpha1 DHPR subunit (Robinson *et al.*, 2000; Jurkat-Rott *et al.*, 2000; Lee *et al.*, 2001). However, theoretically any gene encoding a protein that has primary regulatory functions in the ECC machinery could be a potential candidate for genetic screening and for identification of genetic defects causing protein dysfunction and which may therefore be involved in MH susceptibility in individuals with no known RyR1 or DHPR mutations.

2   The aim of the project

The aim of this study is to elucidate the relationships between susceptibility to MH (MHS) and genes encoding skeletal muscle Ca^{2+} channels and their accessory proteins. The *RYR1* screening project has, however, confirmed that up to 30% of MHS patients carry no *RYR1* variants. Currently, genomic DNA from a panel of 50 independent MHS patients in whom *RYR1* variants have been excluded is available for sequencing of other potential candidate genes. Variants in *CACN1AS* have also been excluded in these samples.

In the present study, *CASQ1* gene which encodes Calsequestrin1 was selected as candidate for DNA sequencing to detect potential causative mutations associated with MH in families with no *RYR1* and *CACN1AS* causative mutations. *CASQ1* gene was selected according to selection criteria for candidate genes which are well-established, including the function of the protein product of the gene, the size of the coding sequence of the gene and the chromosomal location of the gene.

2.1   Strategy for selecting candidate gene

Making the selection of candidate genes for sequencing is a critical step in this project because there are several genes that may contribute to the intracellular Ca^{2+} release mechanism and to malignant hyperthermia. Therefore a proposed strategy for selecting candidate genes was established in the present study according to the following factors:

1. The function of the protein encoded by the gene and whether it could be involved directly or indirectly in the pathophysiology of MH.

2. Protein interaction with RyR1 and/or DHPR proteins.

3. The size of the gene being selected, giving priority to the smallest.

4. Evidence in the literature that the protein or its homologue may be involved in cardiac muscle dysfunctions.

5. Evolutionary conservation.

6. Expression specificity (skeletal muscle).

2.2   Selection of candidate gene

DNA sequencing was carried out to elucidate the genetic variants in the 30% of MHS families with no *RYR1* or *CACNA1S* mutations (Robinson *et al*., 2006). Calsquestrin1 gene was selected according to the selection criteria described earlier, namely involvement in the Ca^{2+} release mechanism and evidence of abnormal Ca^{2+} release in transgenic knock out animal models, mutations in cardiac homologues. Accordingly four exons in Calsquestrin1 (*CASQ1*) were selected.

2.3   Calsequestrin1

In addition to the critical function of skeletal CASQ1 in the Ca^{2+} release mechanism through modulating RyR1 activity, *CASQ1* was selected as candidate gene because it is the major Ca^{2+} storage protein in the sarcoplasmic reticulum. Furthermore, exons 5, 6, 8, and 9 in *CASQ1* were selected for DNA sequencing due to their similarity with their homologous exons in cardiac Calsequestrin2 (*CASQ2*). Sequence homology of the selected exons with their corresponding cardiac homologous sequences shows high similarity in DNA sequence predicting a functional value of these sequences (Figure1). Furthermore, these exons were selected for sequencing due to the detection of mutations which alter the calcium release mechanism in their homologue in human cardiac *CASQ2*. Mutations associated with abnormal Ca^{2+} release in the SR of the cardiac *CASQ2* were detected in exon 9 (De la Fuente *et al*., 2008) and exon 6 (Kirchhefer *et al*., 2010). Mutation D307H located in exon 9 of *CASQ2* is associated with impaired SR Ca^{2+} handling and causes complex ventricular arrhythmias in mice (Viatchenko-Karpinski *et al*., 2007). In addition, it has been demonstrated that mutation p.P308L (c.923C>T), in exon 9 of *CASQ2* is associated with catecholaminergic polymorphic ventricular tachycardia (CPVT) which is a cardiac disorder caused by abnormal calcium release in the SR (De la Fuente *et al*., 2008). Moreover, mutation K206N in exon 6 of *CASQ2* is associated with altered cellular calcium handling (Kirchhefer *et al*.,

2010). In addition, KO mice model lacking *CASQ1* demonstrate abnormal excitation contraction coupling and MHS like symptoms, suggesting skeletal *CASQ1* as candidate gene for MH (Protasi *et al.*, 2009).
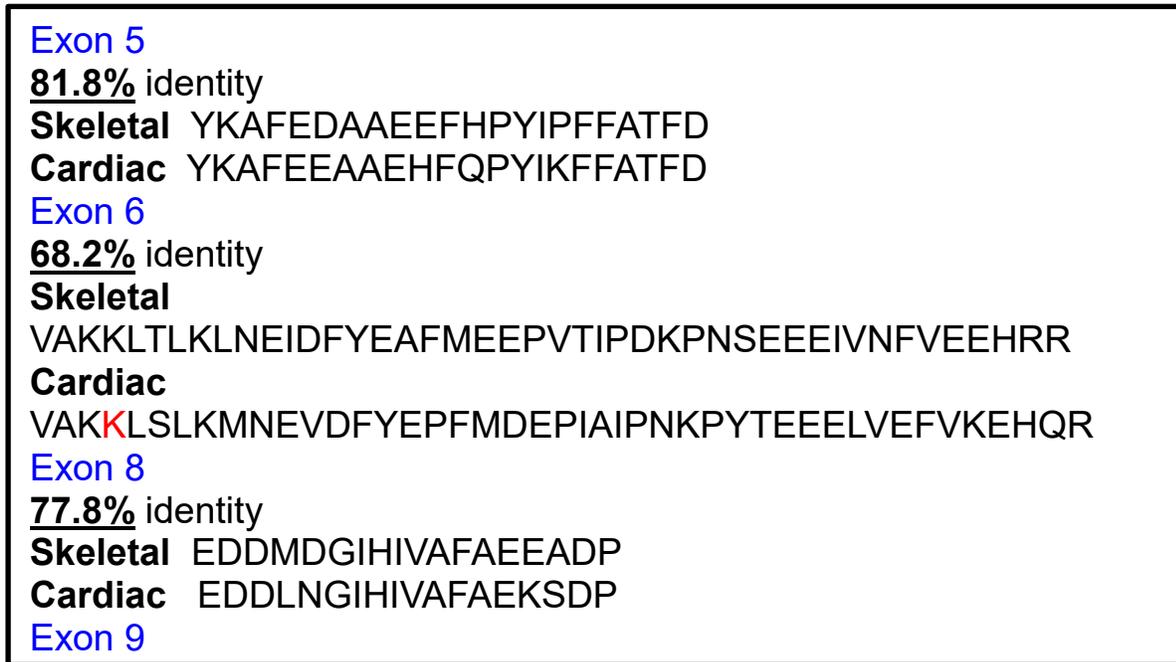
<div style="border:1px solid black; padding:10px">

Exon 5

81.8% identity

Skeletal YKAFEDAAEEFHPYIPFFATFD

Cardiac YKAFEEAAEHFQPYIKFFATFD

Exon 6

68.2% identity

Skeletal

VAKKLTLKLNEIDFYEAFMEEPVTIPDKPNSEEEIVNFVEEHRR

Cardiac

VAKKLSLKMNEVDFYEPFMDEPIAIPNKPYTEEELVEFVKEHQR

Exon 8

77.8% identity

Skeletal EDDMDGIHIVAFAEEADP

Cardiac EDDLNGIHIVAFAEKSDP

Exon 9

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Figure 1. Sequence alignments of the selected regions of Calsiquestrin1.

Alignments of the sequences of exons 5, 6, 8, and 9 of skeletal *CASQ*1 and their homologues in cardiac *CASQ*2. Highlighted amino acids indicating the location of previously indentified mutations in *CASQ2* associated with impaired Ca^{2+} release in the SR.

3   Materials and methods

3.1   Patient samples

DNA samples were obtained from patients who were positive for IVCT and negative for all causative mutations in *RYR1* and DHPR α1S subunit (*CACNA1S*) genes according to the European Malignant Hyperthermia Group (EMHG). Genomic DNA was extracted from EDTA-blood samples, which were from 50 Malignant Hyperthermia Susceptible patients who were referred to the Section of Translational Anaesthetic & Surgical Sciences, Malignant Hyperthermia Unit, S[t] James Hospital, University of Leeds.

3.2   Methods

3.2.1   Genomic DNA extraction

DNA was extracted from whole blood samples in EDTA. The samples were collected and extracted as a part of the continuous routine protocol performed in the MH research programme in the Malignant Hyperthermia Unit, S[t] James Hospital, University of Leeds.

3.2.2   Polymerase chain reaction amplification (PCR)

Genomic DNA fragments of the selected exons of Calsequestrin1 were amplified by PCR in a 20 µl PCR reaction mixture. The genomic sequence of skeletal muscle Calsequestrin1 (*CASQ*1) consists of 11 exons with high homology with the 11 exons of the cardiac muscle Calsequestrin2 (*CASQ*2). For the purpose of sequencing, four exons were selected in *CASQ* 1. Primers were designed to amplify exons 5, 6, 8, and 9 of *CASQ1*. One pair of primers amplified exons 5 and 6 as well as exons 8 and 9.

Table 1. Primer pairs and optimal PCR conditions for amplifying exon 5 & 6 and exons 8 & 9 sequences of Calsequestrin1. The amplification includes the flanking intronic regions. The reaction utilized 1.5 mM of $MgCl_2$ for both primer pairs.

Exon	Primer Sequence (5' to 3')	Products Size (bp)
5 and 6	F: TGGTTTCCCCAGAGACTGAC R: GAGAGGGTTGCCCTTGGT	630
8 and 9	F: TTCTGGGCTCCTCAACTTCA R: GTCCAAGGCCCCACCTTA	500

3.2.3   DNA Sequencing

DNA direct sequencing (ABI 3100 Genetic Analyser, Applied Biosystems) according to the Sanger chain termination technique was used for DNA sequencing (Sanger *et al*., 1977). The sequencing was performed in-house at the University of Leeds, Leeds Institute of Molecular Medicine sequencing services. The coding regions of all the selected candidate genes were sequenced to detect any genetic variants. The sequencing was carried out using the BigDye terminator sequencing kit from Applied Biosystems, according to the manufacturer's instructions.

4   Results

4.1   Analysis of calsequestrin1 sequence

DNA sequencing for exons 5, 6, 8 and 9 DNA of the *CASQ1* gene and flanking sequences were performed successfully. DNA sequence analysis results of the selected exons demonstrated that no mutations or any novel genetic variants were identified in MHS individuals.

5   Discussion

Molecular genetics of MH research is developing and improving with the aim of developing a more convenient (i.e., less invasive) diagnostic test to ultimately replace the IVCT test. It is

hoped that genetic testing for MH diagnosis may be further facilitated by increasing the number of causative mutations known to be associated with the phenotype in addition to the two genes up to now associated with the disease, *RYR1* and *CACNA1S*. Linkage studies and DNA sequencing outcomes exclude the association between MH and the RYR1 or CASCAS1 in approximately 30% of the MH susceptible population. In addition, MH genotype/phenotype discordance which indicates the contribution of several interacting genes in MH even in families with linkage to RYR1 loci demonstrate that susceptibility to the disease is complex. Taken together, these findings indicate a clear genetic heterogeneity of inheritance of the MH phenotype, which opens doors to identification of new potential candidate gene(s) linked to the phenotype.

Identification of modulator genes and their causative mutations associated with MH susceptibility is recommended in understanding MH susceptibility, even in the presence of RYR1 variants (Jurkat-Rott *et al.*, 2000). Robinson *et al.*, (2000) have emphasised the importance of other mutations in modifier genes in MHS individuals with functionally weak or insufficient RYR1 mutations to express the MHS phenotype. Approximately 5-10% phenotype/genotype discordance between IVCT and the presence or absence of causative mutations was observed in some pedigrees (Robinson *et al.*, 2003b). One explanation of this discordance is the involvement of different mutations in different genes (haplotypes) for susceptibility to MH.

In the present study, four selected exons of Calsequestrin1 were selected as candidates for DNA sequencing to screen potentially MH causative mutations. The selection process was according to our established "rational judgment" criteria based on several factors. This strategy of selection is an application of a candidate gene approach to select a potential candidate gene for MH susceptibility based on the biological characteristics of the selected genes (Kwon and Goate, 2000). Establishing such criteria is critical part of the entire project and therefore the selection criteria were chosen according to several biological factors. These investigations enable us to establish a comprehensive list of several potential candidate genes and their characteristics and possible contributions to the Ca^{2+} release mechanism.

Calsequestrin1 was targeted in our study as the major storage protein in the SR and the four exons in *CASQ1* were selected because of their sequence homology with the cardiac homologue *CASQ2* and because pathogenic mutations in these exons in *CASQ2 are* associated with abnormal SR Ca^{2+} release in the SR of cardiac *CASQ2* (Viatchenko-Karpinski *et al.*, 2007). *CASQ1* has been proposed as a candidate gene for MH by other groups (Protasi *et al.*, 2009). However, DNA sequencing analysis of the four screened exons identified no novel sequence variants or causal mutations. However, database SNPs were confirmed in these DNA sequences. Therefore DNA sequencing of the remaining seven exons representing the entire coding sequence of *CASQ1* may be fruitful to detect MH potential causative mutations.

Previous studies have investigated MH susceptibility and found genetic heterogeneity and unclear MH susceptibility in approximately 30% of MH families without mutations in *RYR1* and *CACNA1S* but leave unanswered questions (Robinson *et al.*, 1998; Robinson *et al.*, 2000; Robinson *et al.*, 2006). In particular, these studies have generally failed to elucidate fully the causes of the discrepancy between MH IVCT phenotype and DNA genotype in approximately 10% of MH susceptible individuals (Robinson *et al.*, 2003b). Some studies have investigated this

discrepancy and the heterogeneity of the MH phenotype by using linkage and association studies (Robinson *et al.*, 2000). However, no study as yet has applied a candidate gene approach for identifying causative mutations in potential candidate proteins which influence the dysregulation of calcium homeostasis.

The findings that mutations of *RYR1* and *CACNA1S* may be absent in MHS individuals and the presence of linkage to other susceptibility loci demonstrate strong evidence for MH heterogeneity and the contribution of several interacting genes from a single or multiple loci in MH susceptibility in these individuals (Monnier *et al.*, 1997; Robinson *et al.*, 1997; Robinson *et al.*, 2000). In addition, the interaction of two different loci to produce the MH phenotype was demonstrated by Monnier *et al.*, (2002), who showed that an MHS individual inherited *CACNA1S* mutation from his father and *RYR1* mutation from his mother. Robinson *et al.*, (2000) indicated the influence of additional genetic loci on chromosomes 3q, 5p and 7q in MH susceptibility in families with *RYR1* linkage. The combination of two or more loci may modulate the phenotype of MH and may result in variability in the severity of the phenotype. Therefore, contribution of several genes with minor phenotypic effects may cumulatively result in the MHS phenotype (Robinson *et al.*, 2000).

In the current study, screening four selected exons of the *CASQ1* coding sequence for MH potential causative mutations was performed in 50 MHS individuals. Nevertheless, no causative mutations were detected in these exons. This is the first time that sequencing of the *CASQ1* gene has been performed in MHS patients who have no *RYR1* or *CACNA1S* mutations. Therefore, sequencing the entire coding sequence of *CASQ1* is suggested as *CASQ1* has been considered to be a new candidate gene for MH and exertional or environmental heat stroke (Protasi *et al.*, 2009).

6 Conclusions

In conclusion, selected exons of Calsequestrin1 demonstrated that no mutations or any novel genetic variants were identified in the UK malignant hyperthermia population. Investigating other candidate genes that contribute to the calcium release unit, e.g. HRC, different subunits of DHPR, and Calmodulin, may lead to the identification and characterisation of novel mutations or to the detection of association with MH susceptibility.

Finally, further linkage studies and DNA sequencing of other putative candidate genes other than *RYR1*, *CACNA1S* and *CASQ1*, are suggested to further elucidate MH phenotype/genotype discordance and MH heterogeneity.

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