Resequencing array for gene variant detection in malignant hyperthermia and butyrylcholinestherase deficiency

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1. Introduction

Two inherited pharmacogenetic disorders of primary interest in perioperative medicine are malignant hyperthermia (MH, MIM# 145600) and butyrylcholinestherase (*BCHE*) deficiency (MIM# 177400). MH, a potentially fatal hypermetabolic reaction triggered in predisposed individuals by volatile halogenated anesthetics, depolarizing neuromuscular blocking agents, or their combination, is apparently inherited as a dominant trait. Preoperative diagnosis of MH susceptibility (MHS) is important for the safety of individuals with family history of anesthesia-induced complications. Despite its strong genetic component, MHS diagnosis requires an *in vitro* contracture test (IVCT) to assesses the sensitivity of biopsied skeletal muscle to halothane and caffeine, while genetic tests can establish susceptibility only in carriers of known pathogenic mutations, about half of the cases [1-3]. BCHE deficiency can cause prolonged respiratory paralysis due to delayed hydrolysis of neuromuscular blocking agents widely used in surgical anesthesia, such as succinylcholine or mivacurium. Low plasma BCHE activity is found in cases of toxin exposure, pathological conditions, or due to low-activity enzyme isoforms encoded by certain alleles of the *BCHE* gene [4]. Identifying these alleles, some of them relatively frequent, can help recognizing genetically determined deficiency and, in addition, track inheritance within a family [5]. It has been suggested that a combination of plasma BCHE activity and *BCHE* genotype is the optimal information to evaluate a patient's sensitivity to succinylcholine [6], although diagnostic guidelines similar to MH are currently lacking.

The MHS phenotype is associated with a number of single-nucleotide variants (SNV) in the ryanodine receptor type I gene (*RyR1,* MIM# 180901), its most prominent candidate gene to date [7], and also in the L-type voltage-dependent

calcium channel alpha 1S subunit gene (*CACNA1S,* MIM#114208) [8, 9] and in additional genetic loci [10]. The currently known pathogenic *RyR1* SNV cluster in exons 39-47 and exons 100-102, the rest spreading across exons 1 through 17. This distribution, perhaps because it seems to outline the topography of functionally critical domains in the RyR1 protein, encouraged genotyping exclusively these regions in suspect probands. Although convenient for practical reasons, this approach however ignores the possibility of additional significant variants in the gene, which in fact might have been missed in the past, as none of the MHS-causative *RyR1* variants accepted by the European MH Group (EMHG; www.emhg.org) can be found in 30-50% of MHS individuals [11-13]. Many other *RyR1* variants have been reported in MHS individuals [14-18], but in many cases their isolated occurrence gathered insufficient evidence to determine their relevance. More complete functional characterization of the *RyR1* and *CACNA1S* genes is needed, and could be achieved if full sequences are obtained from individuals in whom phenotypic information is available.

Because of their large size, full-size sequencing of these genes is laborious by traditional sequencing, but amenable with targeted or exome next-generation sequencing (NGS) [19-21], which may in addition add novel genomic insights to MHS and inherited myopathies [22, 23]. The value of NGS for variant discovery is undeniable, but as diagnostic tool it implies costs, specialized bioinformatics and data storage infrastructure that need careful evaluation. Also, objective criteria for data quality assessment are lacking, and data interpretation can be difficult as no reference is available. Finally, although the per-base accuracy of NGS is generally high, it is not error-free: coverage gaps are known to occur, and variant call bias exists depending on the library preparation method, platform, and analysis algorithm used [24-28]. For

example, using amplicon-targeted Illumina sequencing of the *RyR1* gene, Fiszer et al. [19] have already observed that coverage of exons 91 and 102 dropped to zero, requiring an additional Sanger sequencing step to obtain the full sequence. Clearly, standards are needed before safe imputation of genetic variants as the basis of MHS, BCHE deficiency or inherited myopathies can be made in clinical settings [29].

An alternative high-throughput platform worth considering for the detection of variants in a panel of selected genes is the resequencing array. These arrays can be custom-designed and interrogate in a single experiment up to 600,000 bases with high fidelity. They are suited for small sample numbers, and have well-established analysis pipelines and less demanding data storage requirements [30-32]. Arrays are a good fit for the detection of known, clinically actionable variants, and have been already successfully implemented in pharmacogenomic trials and clinical practice as a cost-effective genotyping tool, particularly in large trials [33]. We present here our initial design, validation and trial application of a resequencing array to acquire full sequence information from the *RyR1*, *CACNA1S* and *BCHE* genes. It may be used as a diagnostic tool for probands investigated for MHS or BCHE deficiency, or neuromuscular disorders like central core disease, minicore disease, King-Denborough syndrome or hypokalemic periodic paralysis [34-37] simultaneously enabling full-sequence screening of the target genes for variant discovery.

2. Material and methods

2.1. Patient selection and sample distribution

Patients were registered in the Swiss MH Investigation Unit for diagnosis of MH susceptibility and gave written informed consent. The study was approved by the regional ethical committee (Ethikkommission Beider Basel, EKBB). A total of 124 subjects were selected for this study, which included 121 MHS and 3 healthy controls with no family history of MH. Susceptibility to MH was diagnosed by the IVCT according to the protocol of the European MH Group. [2] Control DNA samples were used to setup all conditions for PCR, capillary sequencing and array hybridization. The MHS samples were divided in 3 groups for different purposes: i) a first group of 56 samples to evaluate the array performance in detecting variations along the whole tiled regions compared with capillary sequencing. In this group, 24 samples were sequenced for *RyR1*, 12 for both *RyR1* and *CACNA1S*, and 20 for *BCHE*. The samples used for RyR1 sequencing were selected from our routine screening and known not to contain any of the common causative mutations; ii) a second group (44 samples) selected to assess the ability of the arrays in detecting specific variations, 16 of them of diagnostic interest. This group contained a total of 29 variants, 24 in the *RyR1* and 5 in the *BCHE* genes. The regions containing such variants were selectively amplified and the resulting fragments pooled in 5 non-overlapping groups, i.e. each pool containing any given variant only once, in order to test as many variants as possible in a single array, and the 5 arrays were run in duplicate; iii) a third group of 21 samples either from relatives of MHS individuals who carried none of the established causative mutations or individuals with a suspected clinical MH episode in whom IVCT information was not available.

2.2. Amplification and capillary sequencing of RyR1, CACNA1S and BCHE genes

Genomic DNA was isolated from either frozen muscle biopsies, or 200 µL EDTA-anticoagulated blood, using the QIAamp® Mini Kit (Qiagen GmbH, Hilden, Germany). Specific primer pairs covering all 3 coding sequences were designed using ExonPrimer, available through the website: http://ihg.gsf.de/ihg/ExonPrimer.html. PCR fragments spanning several exons ranged from 400 to 6400 bp. A total of 22, 12 and 4 primer pairs were necessary to cover all exons of *RyR1*, *CACNA1S* and *BCHE* genes, respectively. PCR was performed in a 25 µl reaction volume with 100 ng genomic DNA, 0.4 µM of each primer, and PrimeSTAR® HS DNA polymerase (Takara Biotechnology, Japan). Two different PCR conditions were set to amplify all regions simultaneously. Purification of PCR products was done with Agencourt AMPure magnetic beads (Beckman Coulter, Beverly, MA, USA). For capillary sequencing, we designed 21 additional primers and used the Big Dye v.3 terminator DNA sequencing kit and an ABI Prism 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence analysis was performed using the Staden Package (http://staden.sourceforge.net).

2.3. Array probe design

The targeted genomic sequences of *RyR1* (NT_011109.17), *CACNA1S* (NT_004487.20) and *BCHE* (NT_005612.17) were checked for the presence of repetitive regions using the web-based tool Repeat Masker (http://repeatmasker.org). According to the protocol, repetitive regions longer than 20 nucleotides were masked and excluded from the probe design algorithm. The probes in the array covered in a tiled fashion the exonic plus 15 bp of flanking, non-coding sequence. Additional

probes covering the sequence of 32 known diagnostic *RyR1* mutations were included to facilitate their detection. These positions were represented twice in the array.

2.4. Array hybridization

The same set of primer pairs described above for sequencing was used in the DNA amplification for array hybridization. Success of the amplification step was examined by agarose gel electrophoresis. A positive internal control, the 1 kb plasmid (IQ-EX) included in the GeneChip Resequencing Assay kit (Affymetrix), was amplified and used in further steps according to the resequencing array protocol, but with some modifications as follows: PCR fragment concentration was measured with Quant-iT Picogreen dsDNA assay kit (Invitrogen, Carsbald, CA). Equimolar amounts of PCR products were pooled and purified by a column-based method (DNA clean and concentrator-25, Zymo Research, Orange, CA). DNAse titration was performed to obtain an optimal fragmentation of the PCR products, and the optimal enzyme concentration, 0.02 U/μg was used for further experiments. Fragmentation and labeling were performed according to the standard protocol and fragments were visualized on a 4% agarose gel stained with ethidium bromide. Washing and staining were done using the GeneChip® Fluidics Station 450, and the arrays were scanned using the GeneChip® Scanner 3000 (Affymetrix).

2.5. Array data analysis and bioinformatics tools

Array data were analyzed using Affymetrix GeneChip Sequencing Analysis software v4.1 (GSEQ). Intensity data files (CEL files) were produced by the array processing software (Command Console Software, Affymetrix) and used by GSEQ to produce files that contained the analyzed results (CHP files). For an optimal performance of

the software, a minimum of 15 arrays is recommended. The software uses a quality score (QS) threshold as a stringency factor that mutually compromises call accuracy and call rate: at high QS (high stringency) fewer false calls are produced, at the expense of lowering the call rate and thus increasing the rate of no-calls (unassigned positions). Reciprocally, at low QS (low stringency), sensitivity is enhanced, increasing call rate and correspondingly the number of false calls. Data were obtained using a diploid model and different QS thresholds.

To analyze the evolutionary conservation of gene positions, multiple sequence alignments at the protein level were performed using ClustalW v1.82 (http://www.ebi.ac.uk/clustalw/).

2.6. Statistical analysis

When comparing base calls from the array data against capillary sequencing, error rate was calculated as the sum of false positive and false negative per total bases analyzed in all samples. We used the following equations to calculate array sensitivity for mutation detection: sensitivity = true positive / (false negative + true positive). Specificity was calculated as: (total bases - no-calls) / (total bases + false positive), total bases being the total bases of the relevant gene multiplied by the number of samples.

3. Results

3.1. Array design

When the sequences of the *RyR1* (106 exons), *CACNA1S* (44 exons) and BCH (4 exons) genes were analyzed bioinformatically, repetitive sequences were identified in exons 35, 76, and 90 of the *RyR1* gene, which led to exclusion of exon 76 and to tile exons 35 and 90 only partially. The remaining exons were covered using 231768 tiled oligonucleotide probes, totaling 28971 bases interrogated in the array (18078, 7528 and 2565 bases for the *RyR1*, *CACNA1S* and *BCHE* genes, respectively). In addition, 800 bases covering 32 MH-causative *RyR1* variations were redundantly tiled owing to their diagnostic relevance.

3.2. Call rate and cross hybridization test

The arrays' basic performance was first evaluated with DNA samples from healthy controls. The quality score threshold (QS) profoundly affected overall call rate, which decreased gradually from QS0 to QS12 (**Figure 1**). To ensure high sensitivity and avoid missing potentially actionable variants, array data were subsequently analyzed at QS values from QS0 to QS4, which yielded call rates from 100 to 87%, respectively. The number of samples analyzed per batch was also assessed, as the software for data analysis recommends a minimum of 15 arrays. Increasing the number of samples beyond 15 did not change the overall call rate: at QS1, call rates were 98.07, 98.06 and 98.15 with 15, 25 and 45 arrays, respectively.

Amplified fragments from the *RyR1* gene in the 3 control samples were hybridized in single arrays in the absence of *CACNA1S* and *BCHE* fragments to assess cross hybridization to non-target probes. At QS0 and QS1, cross hybridization averaged 3.1% and 2.1% respectively, decreasing further at higher QS values (**Table 1**), and

was due mainly to consistent binding to a few exons with call rates exceeding 8% (exons 1, 19, 31 and 42 of *CACNA1S* and 4 of *BCHE)*. In the other regions, cross-hybridization was very low, with median values of 1% at QS0.

3.3. Array performance in the whole tiled regions

The variant detection performance of the arrays was tested using DNA from MHS and *BCHE*-deficient probands, first comparing it with capillary sequencing calls across the tiled gene regions. Capillary sequencing of control samples identified 134 known SNV in the 3 genes in 37 coding positions. In the first group of MHS individuals (n=56), we recognized a total of 847 SNV, which included 493 in 92 coding positions (386, 63 and 44 in the *RyR1*, *CACNA1S* and *BCHE* genes, respectively) in both homozygous and heterozygous form. The missense variants in *RyR1* and *CACNA1S* genes found in these samples are listed in **Table 2**.

The same samples were then analyzed in a set of 36 arrays. Of the 493 SNV detected by sequencing, we found 411 with the arrays analyzed at QS1, localized in 77 coding positions, while 82 SNV mapping to 15 coding positions (9 in the *RyR1*, 3 in the *CACNA1S*, and 3 in the *BCHE* genes) were not detected and thus considered false negatives (**Table 3**). At QS0, 421 SNV were detected and 72 remained no-calls. By decreasing the stringency from QS4 to QS0, false negatives decreased twice for *RyR1*, three times for *CACNA1S* and it remained similar for *BCHE.* From a comparative analysis with capillary sequencing, we obtained sensitivity values of 0.85, 0.87 and 0.66 for the *RyR1*, *CACNA1S* and *BCHE* genes at QS1. Inevitably, lowering the QS threshold increased the number of false positive calls, most of them in the large *RyR1* gene. However, because the number of correctly identified (neutral) bases is very high (e.g. in *RyR1*: 18078 x 36 samples = 650808 bases called), a high specificity of 0.99

for all genes was obtained (**Table 3**). Call error rates decreased from QS0 to QS4, more sharply for RyR1, due to the large decrease in false positives, more gradually for *CACNA1S* and *BCHE* (**Figure 2**).

Handling of the data files was facilitated with Perl scripts to translate the crude output of GSEQ to readable tables detailing the genomic and translation positions and to link to public databanks. These tables showed the localization of false positives and known polymorphisms as recurrent clusters along the tables. Based on our analysis, we decided to use QS1 in further experiments and QS0 for single exons with difficult regions such as those with high GC-content and short repeats. Indeed, 5 *RyR1* exons contained such difficult regions: exons 49, 51 and 70, in which we observed false negatives more often, whereas exons 28 and 46 showed a relatively high number of false positives when analyzed at QS1.

3.4. Array performance in the detection of specific variations

To judge the ability of the arrays in detecting variants at specific positions, including many of diagnostic significance, we gathered 44 DNA samples known to carry a total of 29 known variants (24 in *RyR1,* and 5 in *BCHE*, **Table S1**) and hybridized to 5 arrays. The entire procedure, starting with DNA preparation, was performed in duplicate to test the reproducibility of the method. By first analyzing the additional probes for detection of pathogenic variants, 16 of the 24 *RyR1* variants represented MH-causative mutations validated by the EMHG [2], and already at QS1, all 16 were detected, two of them (c.742G>A and c.1021G>A) reported as homozygous. Interestingly, the simultaneous presence of c.8189A>G and c.8190T>C altered the detection of the c.8189A>G variant, being reported as homozygous. Three variants (c.1630G>T, c.7361G>A and c.7372C>T) were shown not to replicate when analyzed

at QS1, but at lower stringency (QS0), the nucleotides were all correctly assigned. The *RyR1* variant c.7360C>T was reported as false positive in one of the two arrays, however this was not the case when analyzed from the whole tiled region. When all the 29 *RyR1* and *BCHE* variants were analyzed using the probes from the whole tiled regions, 26 were detected in both arrays. The *BCHE* variant c.1699G>A was missed in one replicate and the *RyR1* variants c.7361G>A and c.9649T>C were missed in both replicates at stringency QS1. *RyR1* variants c.742G>A, c.1021G>A and c.1630G>T were called as homozygous in the array while all variants were heterozygous by capillary sequencing.

3.5. Trial application of the arrays to samples of unknown genotype

The protocol defined from the previous results was applied to test samples from MHS individuals of either unknown genotype, or in whom no pathogenic variant had been previously identified. In this third group (n=21), we detected a total of 332 variations with the resequencing array, corresponding to 103 positions in coding regions, which were then assessed by capillary sequencing. From the 103, 39 were not detected (thus, were considered false positives), 51 were common SNV and 13 were missense variants in the *RyR1* and *CACNA1S* genes, some of them not reported before (**Table 4**); those previously registered in the NCBI database had not been linked to any disorder, most were found in single patients, and all in heterozygous form. Most of the novel missense variants encoded aminoacid changes in positions highly conserved across species (**Table S2**), and are therefore potentially pathogenic. In addition, we extracted the *CACNA1S* array data from 24 patients of the first group, in whom only the *RyR1* was sequenced, therefore their *CACNA1S* genotype was unknown. Data analysis in the whole tiled region revealed 3 added missense SNV that were verified

by capillary sequencing. The variant c.5550C>A was found in a patient already carrying c.7007G>A in the *RyR1* gene.

4. Discussion

Genetic and phenotypic heterogeneity is a common source of uncertainty in the diagnosis of several inherited musculoskeletal disorders, as well as in investigating their etiology. High-throughput sequencing can help outline the underlying genomic features in these disorders through variant discovery in many genes simultaneously [38, 39], but the ultra-high output poses limits to its practicability as a diagnostic tool [24-29]. For diagnostic purposes, full sequencing of a panel of firmly established gene candidates can be adequately handled with resequencing arrays, which have already been successfully applied in clinical trials[33]. We developed the POPS array for the detection of variants in the *RyR1, CACNA1S* and *BCHE* genes, known to contribute to acute, life-threatening perioperative hazards such as MHS and BCHE deficiency. Although the MHS trait is genetically heterogeneous, involving probably more than SNV in two genes [10], focusing on a few known genotype checks in the main candidate gene *RyR1* has already been a successful strategy to identify 50-70% of susceptible individuals. Over time however, selective genotyping inevitably leads to stagnated, fragmentary knowledge of MHS etiology. Array screening of these genes can be used to investigate not only known *RyR1* variants but also new variants in investigations of MHS and other inherited myopathies [40-42]. This alternative platform provides, in addition, an orthogonal method that can assist in the implementation of new sequencing technologies.

In our validation experiments, low cross-hybridization and call specificities higher than 0.99 indicated a high yield of correctly assigned bases. To obtain the maximal diagnostic sensitivity, stringency thresholds were adjusted to minimize the number of false negative calls, at the expense of higher false positive rates. It is important to realize that the use of capillary sequencing as reference method, which is itself prone to false negatives by allelic exclusion [22, 43, 44], may have flagged as false positives legitimate variants found only by the arrays. In any case, false positives can be examined later in confirmatory tests. To investigate the source of the more concerning false negatives, we found diploid representation in 92% of the amplified regions by Pacific Biosciences RSII sequencing (experiments not shown), which included GC-rich regions such as exon 91, so allelic dropout was probably not a significant contributor to false negatives. Rather, sequence-specific effects like regions with short repeats, where false and blank calls were observed more recurrently, are likely culprits [45]. Probe redesign with *in silico* modeling can improve call accuracy at these regions in upcoming versions of the array.

Nevertheless, in its current form, optimizing the stringency for high sensitivity, it was possible to correctly detect 28 out of 29 selected *RyR1* variants, including all the 16 known MHS-causative variants tested. Thus, the arrays allow a first screening focused on the diagnostic variants to obtain information from 50-70% of patients. A second, more exploratory analysis of the whole tiled regions can be performed to verify the diagnostic findings, and to investigate the rest of the sequences for new variants. Since 2015, genetic screening is recommended by the EMHG as the first investigation to be carried out in certain MHS probands [2], and positive identification of an established pathogenic *RyR1* variant does establish diagnosis, circumventing the burden of an invasive contracture test. In accordance

with these guidelines a negative genetic test result (e.g. absence of a pathogenic variant) should be followed by an IVCT. This strategy ensures maximal patient safety, as the sensitivity of the IVCT is almost 100%, rendering a false negative diagnosis extremely unlikely.

In our pilot application of the arrays for variant discovery, we identified 16 rare non-synonymous *RyR1* and *CACNA1S* variants, 4 of them novel, in a group of samples from MHS individuals with no known pathogenic variants. GERP scores of 4-5 were found for some of these variants, and high conservation seen in mammalian homology alignments indicated damaging potential. Correlation with phenotype within family members of these carriers was not yet possible, but should help determine whether any of these variants could be of any consequence for MHS. This trial illustrates how, unlike selective genotyping, systematic array screening produces at no extra effort an information record useful to track variants that may be confirmed as deleterious in the future.

Either via arrays or NGS, comprehensive genotyping of individuals investigated for MHS, BCHE deficiency, or inherited myopathies is crucial to improve the functional annotation of the vast amounts of variants listed in public databases. Only in the *RyR1* gene, 1837 missense, 2754 nonsense and 43 frameshift variants are currently listed in dbSNP, but the majority of them are of unknown significance as most originate from control individuals lacking phenotypic information. The subclinical nature of MHS and some inherited myopathies restricts the functional information of variants to the centers where probands are tested. Current trends suggest that broader genetic monitoring of patients is underway [46], and the dissemination of new associations between genomic information and phenotype should expand the reference information about pathogenicity of variants,

their frequency and their geographical distribution. This will increase the chances for MHS individuals to be identified without invasive tests, and provide support in the diagnosis of patients with rare myopathies bearing variants for which no reliable information is currently available.

In summary, array screening of *RyR1, CACNA1S* and *BCHE* genes for suspect individuals of MHS and BCHE deficiency and their family members is a step forward towards a better understanding of the core genetics of these disorders at a reasonable cost and labor, setting the stage for improved diagnostics in the future. The POPS array could be used as a universal genotyping tool for different neuromuscular disorders, offering the possibility to simultaneously screen established variants and discover new ones in disease genes of established relevance.

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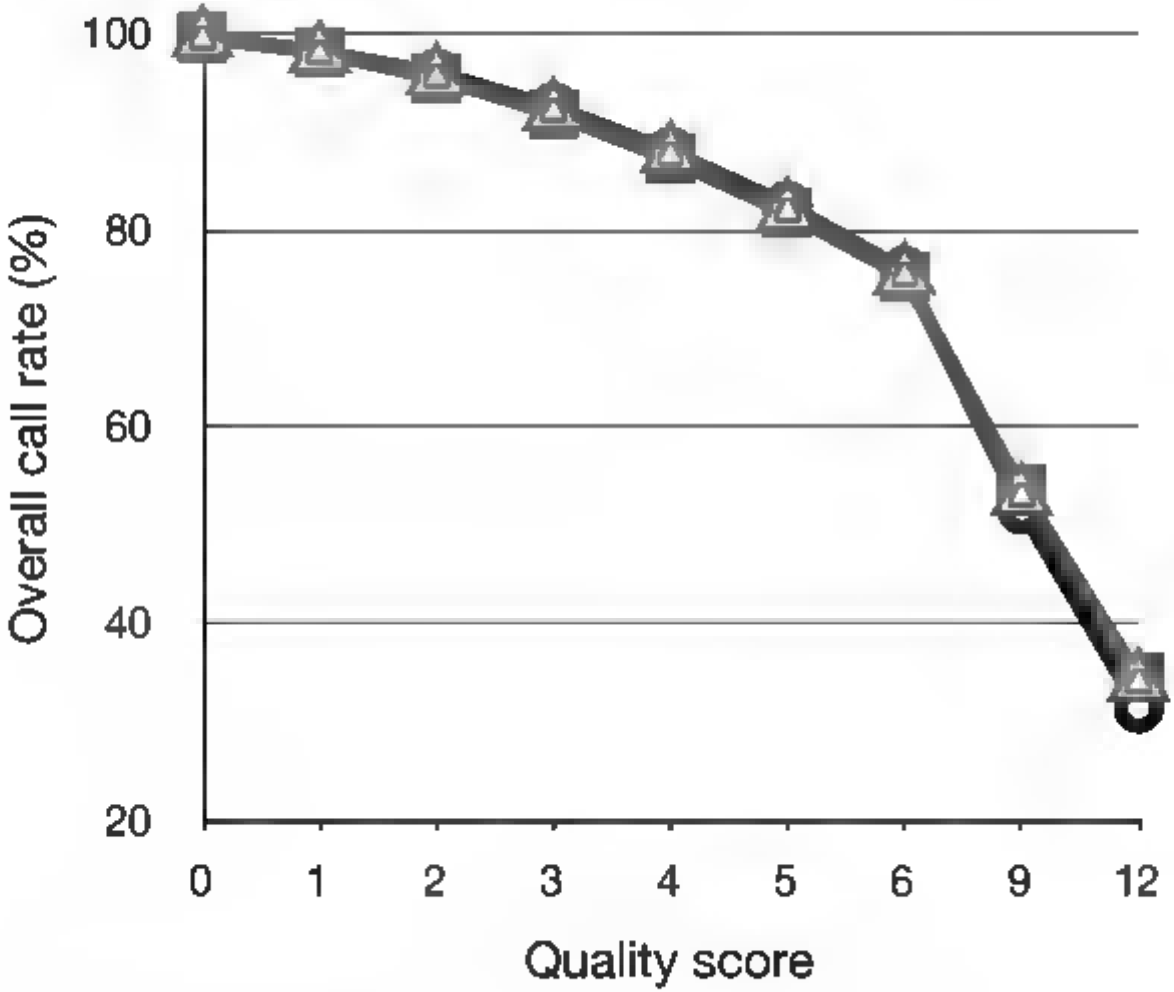
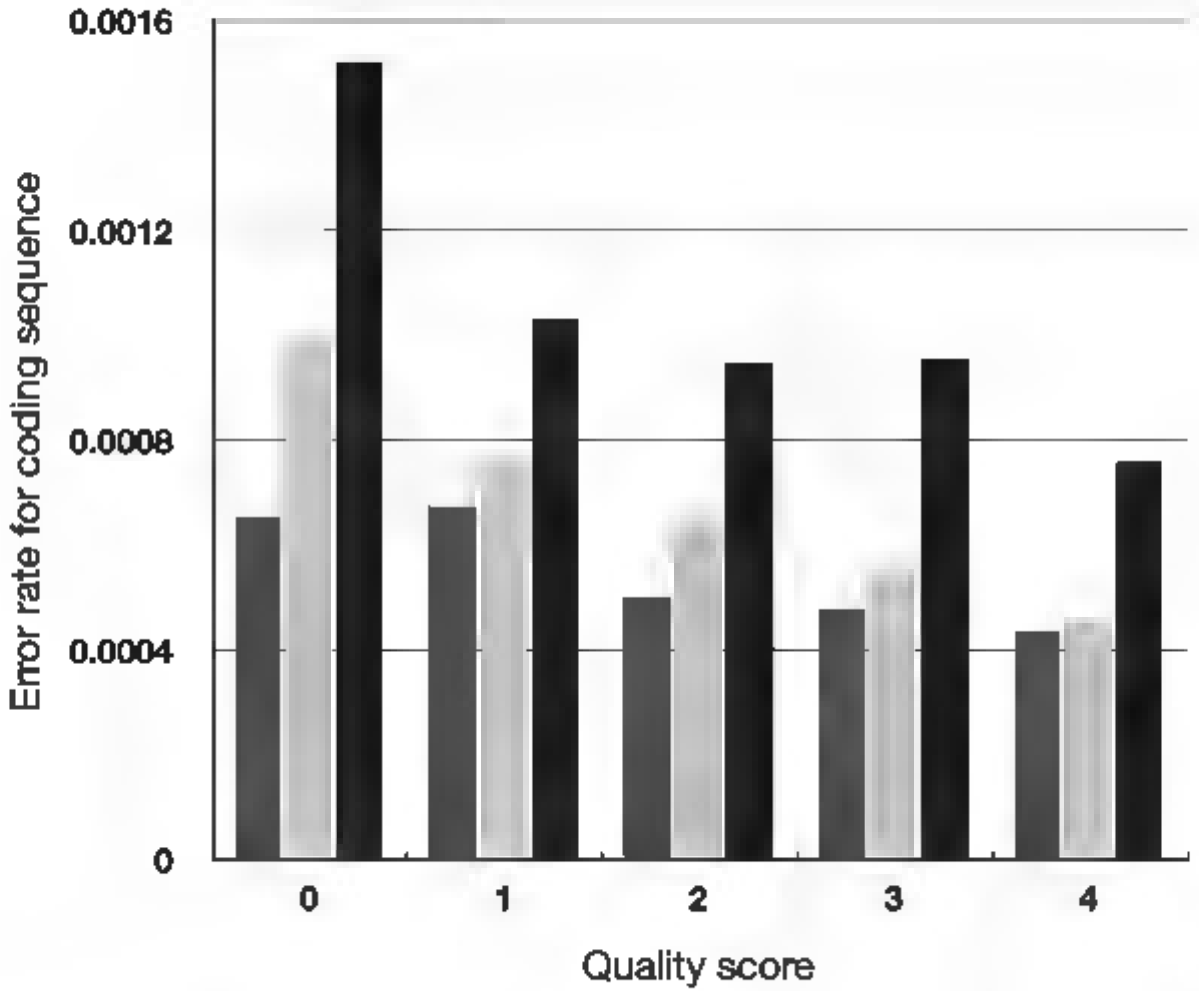
Figure legends

Figure 1. Overall call rate at different quality score (QS) thresholds. Circles represent call rates corresponding to *RyR1*, squares to *CACNA1S* and triangles to *BCHE*.

Figure 2. Genotyping call error rates for the 3 coding regions at QS thresholds QS0 to QS4. Error rate was calculated as the sum of false positives and false negatives per total bases interrogated. Black bars=*RyR1*; dark grey bars: *CACNA1S*; light grey bars=*BCHE.*


100
80
60
40
20
Overall call rate (%)
0
1
2
3
4
5
6
9
12
Quality score


0.0016
0.0012
0.0008
0.0004
0
Error rate for coding sequence
0
1
2
3
4
Quality score

Tables

Table 1. Cross-hybridization test

QS	Overall call rate (%)	Hyb. control (%)	Cross-hybridization (%)*			
			Mean	Median	Max	Min
0	100	100	3.1	1	19.8	0
1	98	99	2.1	0.6	11.5	0
2	96	98	1.6	0	8.9	0
3	92	95	1.2	0	7.3	0
4	87	90	0.9	0	6.3	0

* Percentage values correspond to called bases respect to the total bases of an exon

Table 2. Missense variations* of *RyR1* and *CACNA1S* detected by capillary sequencing in 56 MHS individuals

Gene	**Exon**	**Nucleotide change**	**Aminoacid change**	**References**
RYR1	34	c.5495G>C	p.Gly1832Ala	rs193922784
RYR1	38	c.6178G>T	p.Gly2060Cys	rs35364374
RYR1	48	c.7648C>G	p.Leu2550Val	rs193922821
RYR1	64	c.9476A>G	p.Asp3159Gly	This study[1]
CACNA1S	4	c.520C>T	p.Arg174Trp	rs772226819
CACNA1S	10	c.1373T>A	p.Leu458His	rs12742169
CACNA1S	11	c.1547C>T	p.Ser516Leu	rs140662085
CACNA1S	12	c.1817G>A	p.Ser606Asn	rs142356235
CACNA1S	38	c.4615C>T	p.Arg1539Cys	rs3850625
CACNA1S	40	c.4973G>A	p.Arg1658His	rs13374149
CACNA1S	44	c.5399T>C	p.Leu1800Ser	rs12139527

* Numbering starts with +1 corresponding to A of the ATG start codon of NCBI accession numbers NM_000540.2 and NM_000069.2 for *RyR1* and *CACNA1S* genes, and aminoacid accession numbers NP_000531.2 and NP_000060.2 for *RyR1* and *CACNA1S*; [1] NCBI rs879074444

Table 3. Genotype calls of the POPS array compared with capillary sequencing

	QS	True positive	False negative	False positive	No-calls	No-call rate ($x10^{-3}$)	Sensitivity	Specificity
RY	0	334	52	939	203	0.3	0.87	0.99
R1	1	327	59	614	276	0.4	0.85	0.99
	2	308	78	539	236	0.4	0.80	0.99
	3	296	90	533	249	0.4	0.77	0.99
	4	285	101	394	263	0.4	0.74	0.99
CA	0	56	7	82	28	0.3	0.89	0.99
CN	1	55	8	61	28	0.3	0.87	0.99
	2	52	11	47	56	0.6	0.83	0.99
A1	3	46	17	33	63	0.7	0.73	0.99
S	4	41	22	19	69	0.8	0.65	0.99
BC	0	31	13	17	22	0.5	0.71	0.99
HE	1	29	15	16	36	0.8	0.66	0.99
	2	29	15	8	36	0.8	0.66	0.99
	3	29	15	7	37	0.8	0.66	0.99
	4	29	15	5	38	0.8	0.66	0.99

Table 4. List of *RYR1* and *CACNA1S* SNV detected by the POPS array in a group of patients with unknown genetic information

Exon	Nucleotide change	Aminoacid change	Samples	References
RYR1				
2	c.122T>C	p.Phe41Ser	1	rs766407858
4	c.338G>A	p.Ser113Asn	1	This study[1]
18	c.2030A>G	p.Gln677Arg	1	This study[2]
20	c.2488C>T	p.Arg830Trp	1	rs142548565
33	c.4711A>G	p.Ile1571Val	2	rs146429605
34	c.5000G>A	p.Arg1667His	1	rs138978909
40	c.6617C>T	p.Thr2206Met	1	rs118192177
43	c.7020C>G	p.Phe2340Leu	1	This study[3]
43	c.7025A>G	p.Asn2342Ser	1	rs147213895
46	c.7354C>T	p.Arg2452Trp	1	rs118192124
66	c.9998C>G	p.Ser3333Cys	1	This study[4]
67	c.10097G>A	p.Arg3366His	2	rs137932199
86	c.11798A>G	p.Tyr3933Cys	2	rs147136339
CACNA1S				
2	c.206C>G	p.Ala69Gly	1	rs12406479
44	c.5550C>A	p.Asn1850Lys	1	rs141556780
44	c.5570G>A	p.Ser1857Asn	1	rs72749169

[1] dbSNP rs879083783, [2] rs878950984, [3] rs199993301, [4] rs878944689